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14. ABSTRACT We hypothesize that immune responses to HER3 will block HER3 mediated endocrine-resistance, provide an effective therapy for endocrine- resistant patients, and eliminate the emergence of resistant clones. The study objective is to generate a clinically applicable HER3 cancer vaccine using advances in vector design that will optimize vaccine performance in clinical settings. Our preliminary data suggests that a vaccine targeting HER3 can generate polyclonal HER3 specific immune responses that have antitumor activity. Our translational studies intend to generate a GMP version of our HER3 vaccine, test its safety and immunogenicity, and then first evaluate whether HER3 vaccination in combination with standard anti-hormonal therapy in an anti-hormonal resistant population leads to increased efficacy of the standard therapy. These studies will support the long term goal of vaccinating against HER3 to prevent resistance. We have generated 4 different Ad5(E2b-)HER3 vectors and confirmed induction of anti-HER3 cellular and humoral immune response and antitumor effect by Ad-HER3 vaccine in mice as preclinical basis for selection of vector construct for clinical use. We have established multiple Tam-resistant ER+/HER3 expressing human breast cancer cell lines and developed HER3 activated pathway signatures using heregulin (HRG) as an activator of the HER3 pathway. These models will be used to identify the protein architecture associated with HER3 pathway activation.					
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1. INTRODUCTION:

Estrogen receptor (ER) positive breast cancer is most the common form of breast cancer, accounting for approximately 70% of all newly diagnosed breast cancer cases. Approximately one-third of women treated with tamoxifen for 5 years will have recurrent disease within 15 years and despite the advent of newer therapies. Resistance to anti-estrogen therapy appears to result from a complex compensatory network. We propose a novel approach to treating breast cancer that has the potential to target multiple pathways for the lifetime of the patient, specifically active immunotherapy directed toward tumor associated antigens evoked by the compensatory resistance mechanisms. We propose to explore a HER3 vaccine that induces polyclonal antibody and T cell responses as it can provide long term anti-HER3 immune responses, which could provide the long term effects needed to prevent the emergence of resistant clones. In addition to the long term protection afforded by vaccination, polyclonal immune responses to a target protein may offer additional benefits. We believe that HER3 may represent a relevant target in endocrine therapy-resistant breast cancer, and a HER3 vaccine may prevent endocrine therapy-resistance by disabling HER3-mediated resistance. Additionally, the HER3 vaccine could potentially be used in combination with endocrine therapy to prevent the onset of therapeutic resistance mediated by HER3 signaling.

2. BODY:

Aim 1: Generation of GMP Ad5(E2b-)HER3 (Y 1-3)

Task 1A: Generate Ad5(E2b-)HER3 and Ad5(E2b-)HER3 C1C2 constructs (Y1, Q1-2)

For Aim 1, Task 1, we have modified the adenovirus construction methods to facilitate the production of the next generation Ad5 vectors with deletion of multiple early genes (E1, E2b, E3). Our previous studies have demonstrated that Ad5(E2b-) vectors are more potent immunogens compared 1st generation Ad even in the presence of pre-existing anti-Ad5 immunity. Adenoviral vectors expressing human HER3 with Ad5(E2b-) platform have been constructed by Dr. Hongtao Guo.

The human HER3 full length cDNA was obtained from OriGene (Rockville, MD). The truncated HER3 extracellular domain (ECD) and HER3 ECD plus transmembrane (TM) sequence were created using HER3 full length as templates in a PCR reaction using primers (see table 1 and figure 1 below).

Table 1: Primers used in construction of truncated Ad5-human HER3

Primer	Sequence
hHER3-F	5'-cagggcgccgcacccatgagggcgaacgacgctct-3'
hHER3-ECDTM-R	5'-acaagcgccgcaggttaaaaagtgccgcccagcatca-3'
hHER3-ECD-R	5'-acaagcgccgcatttatgtcagatgggtttgcccgatc-3'
hHER3-ECDC1C2-R	5'-acaagcgccgcattgtcagatgggtttgcccg-3'

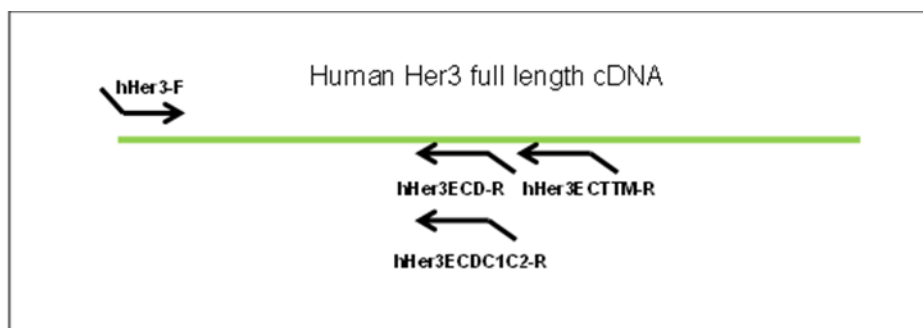


Figure 1. Schematic representation of primers binding site at human HER3 full length cDNA

Briefly, full length HER3 cDNA and the PCR product are cut by restriction enzyme Not I and subcloned into Not I digested pShuttle-CMV or pShuttleCMV-C1C2 plasmid. Confirmation of correct insert of the full length and truncated DNA within pShuttle-CMV or pShuttle-CMV-C1C2 was confirmed by DNA sequence.

The pShuttle-CMV-HER3-FL, pShuttle-HER3ECD, pShuttle-HER3ECDTM and pShuttle-HER3ECDC1C2 were then linearized using digestion with Pme I, recombined into linearized (E1-,E2b-,E3-) serotype 5 pAd construct in BJ 5183 bacterial recombination-based system (Stratagene), and propagated in XL10-Gold Ultracompetent cells (Stratagene). Complementing C7 cell (which express E1 and E2b) were used to produce high titers of these replication-deficient Ad5 vectors, and cesium chloride density gradient was done to purify the Ad5-vectors. All Ad vector stocks were tested for replication-competent adenovirus via PCR-based replication-competent adenovirus assay.

We have generated our next generation human HER3 (E1-, E2b-, E3-) Adenovirus vectors as follows:

1. Ad5 (E2b-)HER3 FL; express human HER3 full length.
2. Ad5 (E2b-)HER3ECDTM; express human HER3 ECD and trans-membrane domain
3. Ad5 (E2b-)HER3ECD; express human HER3 ECD
4. Ad5 (E2b-)HER3ECDC1C2; express human HER3 ECD and C1C2 domain

Initial testing of Ad-HER3 vector was conducted by measuring HER3 specific immune responses in BALB/c mice.

We have established a HER3 prevention model using JC-HER3 mouse mammary tumor cells in BALB/c mice. JC murine breast cancer cell line (BALB/c strain) was transfected with human HER3 using lentiviral vector. Immunogenicity and vaccine efficacy of Ad vectors were determined in BALB/c mice by assessing preventive effect of HER3 vaccination (Figures 2-5).

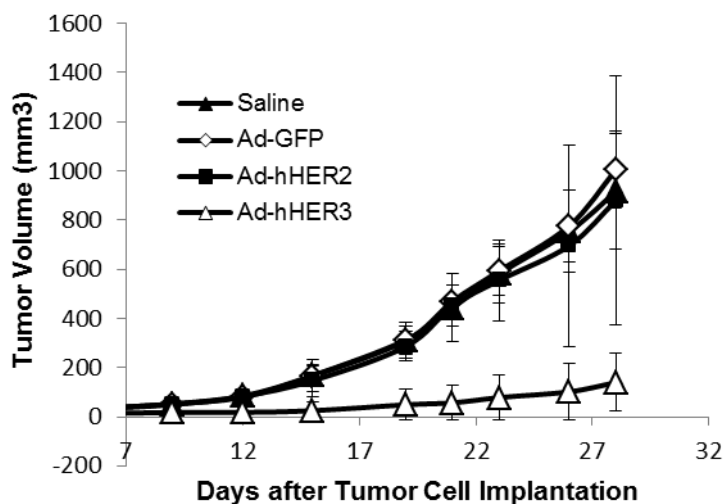


Figure 2. Ad-hHER3 vaccine inhibits JC-HER3 tumor growth. BALB/c mice were vaccinated twice (day-18, day-4) via footpad injection with Ad-GFP, Ad-hHER2 or Ad-hHER3 vectors (2.6×10^{10} particles/ mouse). Four day after boosting, at day 0, each mouse was implanted with 1,000,000 JC-HER3 mouse mammary tumor cells expressing human HER3. Tumor volume was measured every 3 days.

As shown in Figure 3, only vaccination with the HER3 encoding vector prevented growth of HER3 expressing tumors.

To confirm the induction of HER3 specific immune response in Ad-HER3 vaccinated mice, we performed ELISPOT assay with splenocytes from vaccine treated mice as shown in Figure 3.

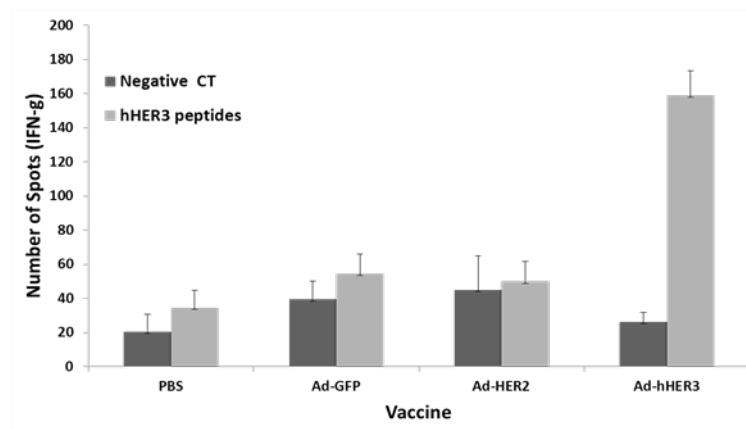


Figure 3. Ad-hHER3 vaccine induced HER3 specific T cell response. Splenocytes (500,000 cells/well) from Ad vaccinated BALB/c mice were collected on day 28 and stimulated with HER3 peptide mix (hHER3 peptides) ($1 \mu\text{g/mL}$ was used; JPT, Acton, MA) or HIV peptide mix (BD Bioscience) as a negative control (Negative CT) and analyzed in a interferon-gamma ELISpot assay.

We confirmed the establishment of anti-HER3 cellular immune response in mice vaccinated with Ad-HER3. We hypothesized that Ad-hHER3 induced anti-HER3 immune response will affect the HER3 expression by tumors grown in mice. Therefore, we tested the HER3 expression in tumor tissue by Western Blot assay, as shown in Figure 4.

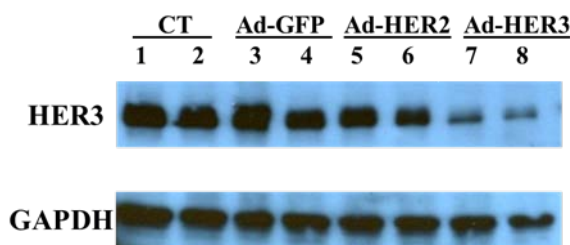


Figure 4. Ad-hHER3 vaccination causes degradation of HER3 on JC-hHER3 tumor. Tumors were isolated from vaccinated and control BALB/c mice (as indicated on figure) and immediately flash frozen. Tissue extracts were prepared by homogenization in RIPA buffer. Equal amounts of protein from each sample were

used to visualize the indicated molecules by immunoblotting.

As shown in Figure 4, immunization with Ad-hHER3 led to a reduction of HER3 expression in the tumors. Immunization with Ad-GFP or Ad-hHER2 did not change HER3 expression by JC-HER3 tumors. We also sought to test for the cell surface HER3 expression by tumors that grew in the HER3 vaccinated mice. Excised tumors were digested with collagenase /hyarulonidase /DNase, and collected tumor cells were stained with PE conjugated anti-HER3 mAb, and analyzed by flow cytometry as shown in Figure 5.

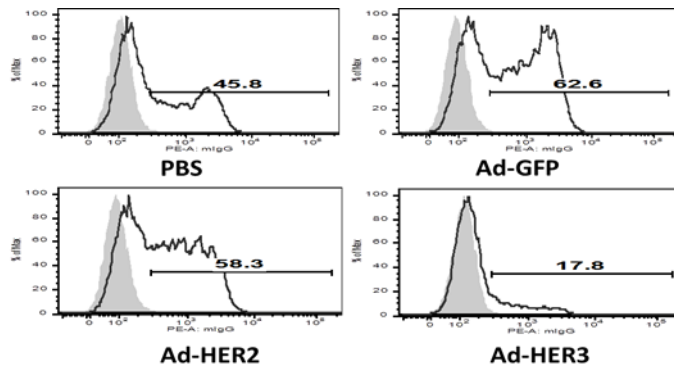


Figure 5. Ad-hHER3 vaccination decreases HER3 expression on JC-hHER3 tumor cells. JC-HER3 tumors were collected from vaccinated and control Balb/c mice (as indicated on figure) at day 28 and pooled by group. The tissues were minced and digested with an enzymatic cocktail (Hyaluronase, DNase, and Collagenase) overnight. After 3 days culture, the cells were harvested and HER3 expression determined by flow cytometry using PE-anti-hHER3 antibody. Solid line: anti-HER3 mAb. Grey histogram:

PE-conjugated IgG.

As demonstrated in Figure 5, the surface expression of HER3 was dramatically reduced in the tumors that did grow in the HER3 vaccinated mice, suggesting the elimination of HER3-positive tumor cells or downregulation of HER3 expression by vaccine induced anti-HER3 immune response.

Aim 1: Generation of GMP Ad5(E2b-)HER3 (Y 1-3)

Task 1B: Preclinical immunogenicity testing of Ad5(E2b-)HER3 (Y1, Q2-3)

Immunogenicity Test in BALB/c Mice:

As described in Task 1A, we generated 4 different adenoviral vectors encoding human HER3 genes, Ad5(E2b-)HER3 FL, Ad5(E2b-)HER3ECD, Ad5(E2b-)HER3ECDTM, and Ad5(E2b-)HER3ECD1C2. To compare the immunogenicity of these vectors in BALB/c mice, female mice (10 mice/group) were vaccinated twice with 2 weeks interval, and human HER3 expressing murine breast cancer cell line (JC-HER3, 1 M cells/mouse) was injected to the flank of mice 4 days later. From each group, 3 mice were sacrificed before tumor cell implantation to collect blood and spleen for immune monitoring. Tumor volume was monitored for the rest of the mice until human endpoint is reached.

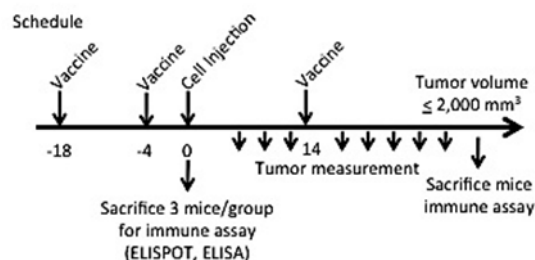


Figure 6. Scheme of Immunogenicity Testing and Antitumor Efficacy Testing. On days -18 and -4, mice were vaccinated with Ad-vectors (2.6×10^6 vp/mouse), and 3 mice from each group were sacrificed for immune assays on day 0. Spleen was harvested for ELISPOT assay, and blood for the test of antibody production. For other 7 mice in each group, JC-HER3

cells were subcutaneously injected to the flank of BALB/c mice. Tumor size was measured until tumor volumes reach 2,000 mm³.

a) Humoral Immune Response: We analyzed established humoral immune responses against HER3 in these mice by flow-based assay.

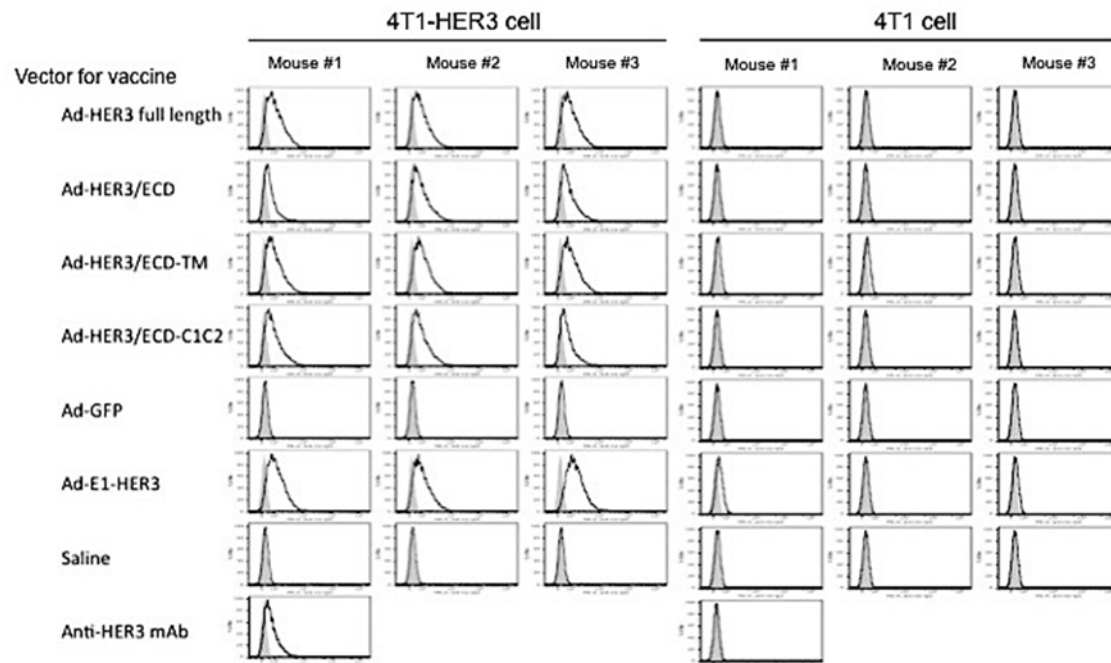
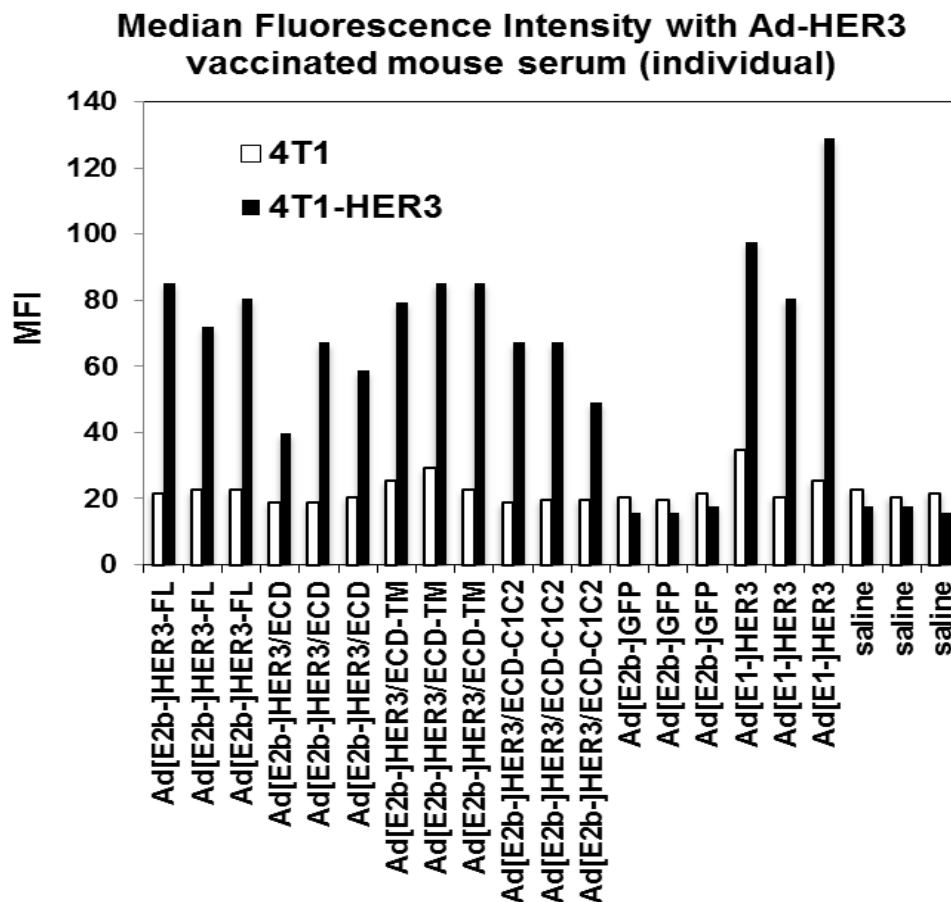


Figure 7. Anti-HER3 antibody levels in the serum of Ad-HER3 vaccinated mice.

Three mice from each group were sacrificed, and serum was collected. 4T1 (HER3-negative) and 4T1-HER3 (transfectant) were labeled with serum (1:100 dilution) and then with PE-conjugated anti-mouse IgG Ab. Open histograms (black line) show staining with mouse serum, and grey histograms show staining without serum (2ndary Ab only).

4T1 (HER3 negative) or 4T1-HER3 (HER3 transfectant) cells were incubated with mouse sera, which were diluted with saline (1:100 dilution), then with PE-conjugated secondary antibody (anti-mouse IgG). Sera from Ad-GFP vaccinated mice were used as negative control, commercially available anti-HER3 mAb as positive control, and mouse serum from Ad[E1-]HER3 vaccinated mice were used for comparison purpose (Figures 7 & 8). Mean Fluorescence Intensity for each serum is shown in the graph below (Figure 8).



Vaccination

Figure 8. Median Fluorescence Intensities for the staining of 4T1 and 4T1-HER3 cells with individual mouse serum.

As shown in the figure 8, Ad-HER3/ECD-TM and Ad-HER3-FL induced slightly stronger anti-HER3 antibody production, and Ad-HER3/ECD was the weakest among 4 newly generated Ad-HER3 vectors.

We further analyzed anti-HER3 antibody level in each serum by cell-based ELISA. 4T1 murine breast cancer cell line (HER3 negative) and human HER3 transfectant (4T1-HER3) were used in this assay. Sera from individual mice were titrated from 1:50 to 1:6400 (Figure 9).

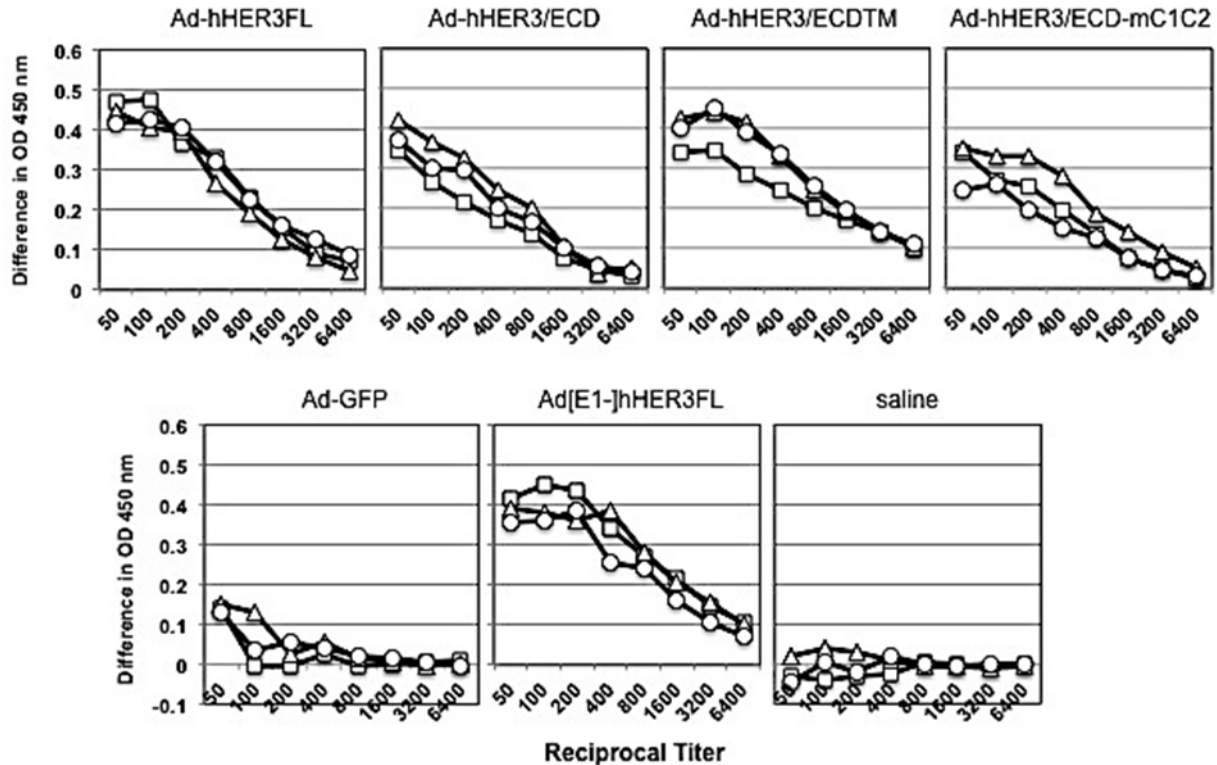


Figure 9: Cell-based ELISA with mouse serum. 4T1 and 4T1-HER3 cells were seeded into 96 well plates. After overnight incubation, cells were washed with buffer, and mouse serum with serial dilutions were added (1:50 to 1:6400) and incubated for 1 h on ice. Then, cells were fixed with 4% formaldehyde, and HRP-labeled Goat anti-mouse IgG (1:1000) was added. After 1 h incubation, washed with PBS 3 times, and TMB was added for 5 min. Color development was stopped by adding H₂SO₄. Differences of OD₄₅₀ values ($=[\text{value for 4T1-HER3}] - [\text{value for 4T1}]$) are shown.

Based on Cell-based ELISA, anti-HER3 antibody production in Ad-HER3 vaccinated mice were confirmed in all mice. These 4 newly made adenoviral vectors showed comparable efficacy in induction of humeral immunity, but Ad-hHER3FL and Ad-hHER3/ECDTM induced slightly higher levels of anti-HER3 antibody and Ad-hHER3/ECD induced slightly lower level.

b) Antigen-specific Cellular Response: We also analyzed antigen-specific cellular immune response by IFN-gamma ELISPOT assay with mouse splenocytes (Figure 10). Splenocytes from each mouse were incubated with HER3 peptide pool (Extracellular domain (ECD), or Intracellular Domain (ICD)), and HIV peptide mix as a negative control, PMA+ Ionomycin as a positive control. As expected, only Adenoviral vectors encoding full length HER3 (Ad5(E2b-)HER3 FL and Ad(E1-)HER3 FL) induced T cell response for the intracellular domain of HER3. T cell responses against the peptide mix of HER3 extracellular domain were variable. Ad-hHER3/ECD-TM induced the strongest cellular response against extracellular domain of HER3. Ad-HER3 virus encoding full length, however, induced only weak cellular response against extracellular domain.

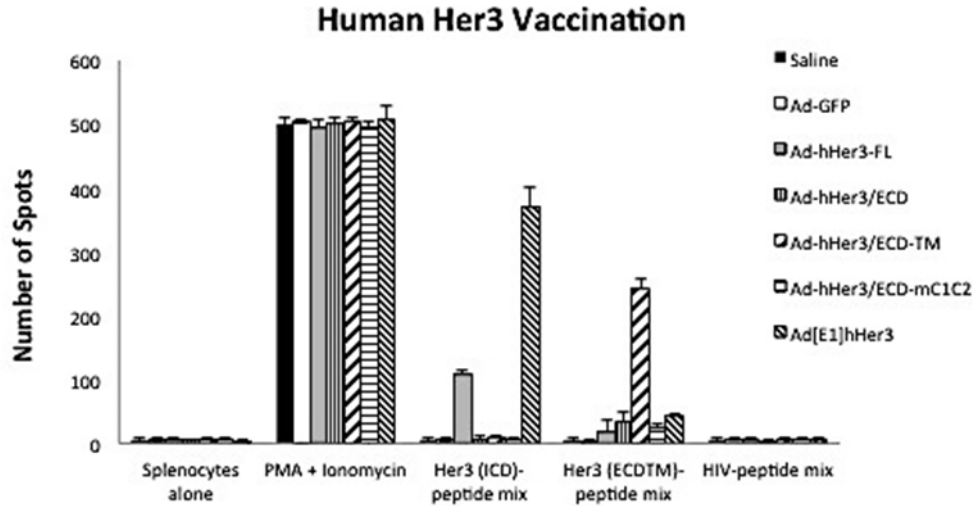


Figure 10. Anti-HER3 cellular response induced by Ad-HER3 vaccination.

Mice were vaccinated with Ad-hHER3-full length(FL), Ad-hHER3/ECD, Ad-hHER3/ECD-TM, Ad-hHER3/ECD-mC1C2, or control Ad-GFP, Ad[E1-]HER3 (2.6×10^{10} vp /vaccination). Two weeks later, vaccination was repeated with the same Ad vectors, and 4 days later, spleen was collected to assess anti-HER3 cellular response. ELISPOT plates were coated with anti-IFN γ mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. Average of 3 mice from each group is shown.

c) Antitumor Response: Tumor growth was measured twice a week until 34 days after tumor cell implantation. Once the tumor volume reached 2,000 mm³ or tumor had ulceration, mice were euthanized. Until day 20, all mice survived and the average tumor volume was calculated for each group and shown in the Figure 11. We are currently making statistical analysis to determine which group showed the strongest antitumor immune response. Our preliminary results for the statistical analysis are shown below.

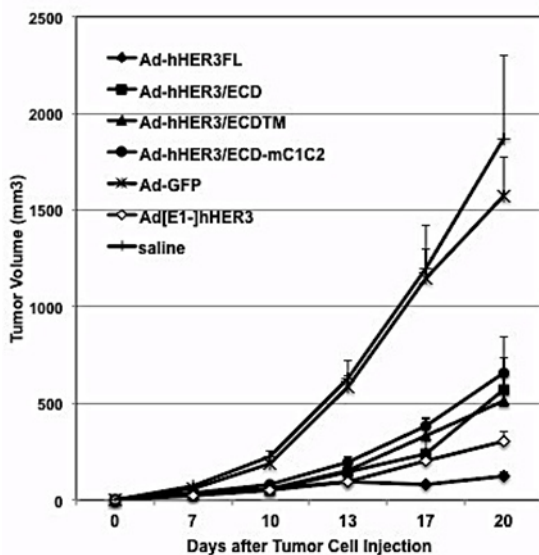


Figure 11. Ad-HER3 vaccine effect on JC-HER3 tumor growth in BALB/c mice.

BALB/c mice were vaccinated twice (day-18, day-4) before and once (day 14) after tumor cell implantation, with Ad[E1-E2b-]hHER3FL, Ad[E1-E2b-]hHER3/ECD, Ad[E1-E2b-]hHER3/ECDTM, Ad[E1-E2b-]hHER3/ECD-mC1C2, Ad-GFP, Ad[E1-]hHER3FL (2.6×10^{10} particles/ mouse) or saline via footpad injection. On day 0, each mouse was implanted with JC-HER3 mouse mammary tumor cells expressing human HER3 (1×10^6 cells/mouse). Tumor volume was measured every 3 days. Error Bar: SE

A mixed model was used to analyze the data. Square root transformation was used for tumor volume to make the relation volume vs time linear and normalize the data. The model results clearly

show that the tumor volume increases with time (Days) for the Saline group. The growth rate of

tumor volume for the vaccine Ad-hHER3FL, Ad-hHER3/ECD, Ad-hHER3/ECD-TM, Ad-hHER3/ECD-mC1C2 are significantly slower than that in Saline, while the difference in the tumor growth in Saline and Ad-GFP is not significant.

Fixed effects: sqrt(vol) ~ groupname * days

	Value	Std.Error	DF	t-value	p-value
(Intercept)	0.4969292	1.9609008	238	0.253419	0.8002
groupnameAd[E2b-]hHER3FL	-0.4177898	2.7731325	42	-0.150656	0.8810
groupnameAd[E2b-]hHER3/ECD	-0.5374032	2.7731325	42	-0.193789	0.8473
groupnameAd[E2b-]hHER3/ECDTM	-0.4758268	2.7731325	42	-0.171585	0.8646
groupnameAd[E2b-]hHER3/ECD-mC1C2	-0.4648125	2.7731325	42	-0.167613	0.8677
groupnameAd[E2b-]GFP	0.0383965	2.7731325	42	0.013846	0.9890
groupnameAd[E1-]hHER3	-0.5313263	2.7731325	42	-0.191598	0.8490
days	1.9160718	0.1107739	238	17.297149	0.0000
groupnameAd[E2b-]hHER3FL:days	-1.3331182	0.1566579	238	-8.509742	0.0000
groupnameAd[E2b-]hHER3/ECD:days	-0.9370645	0.1566579	238	-5.981598	0.0000
groupnameAd[E2b-]hHER3/ECDTM:days	-0.9100369	0.1566579	238	-5.809072	0.0000
groupnameAd[E2b-]hHER3/ECD-mC1C2:days	-0.7315133	0.1566579	238	-4.669496	0.0000
groupnameAd[E2b-]GFP:days	-0.0986146	0.1566579	238	-0.629490	0.5296
groupnameAd[E1-]hHER3:days	-1.0963448	0.1566579	238	-6.998338	0.0000

All newly made Ad-HER3 vectors showed antitumor activity, inhibiting the JC-HER3 tumor growth in BALB/c mice compared to control groups (saline injection). Especially, Ad-hHER3-FL demonstrated the strongest inhibitory effect for tumor growth after day 17. We also plotted individual tumor growth in each group (Figure 12).

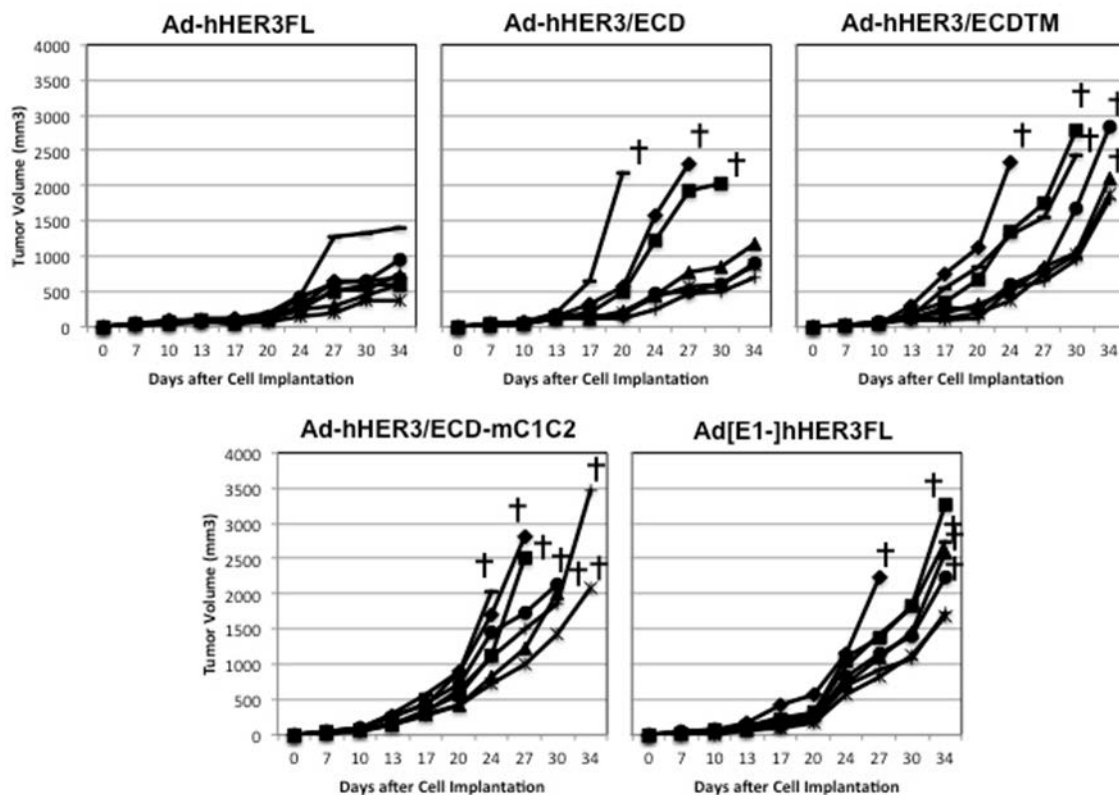


Figure 12. Ad-HER3 vaccine effect on JC-HER3 tumor growth in BALB/c mice. Individual tumor growth. Mice were euthanized when tumor volume reached 2000 mm³ or had ulceration on the tumor.

By day 34 after tumor cell injection, 3 mice in Ad-hHER3/ECD, 5 mice in Ad-hHER3/ECD-TM, all 7 mice in Ad-hHER3/ECD-mC1C2 group died, but no mice died in Ad-hHER3-FL vaccine group. First generation Ad-viral vector, Ad[E1-]-hHER3-FL showed strong tumor growth inhibition at earlier days, but after day 24, tumors started growing faster, and by day 34, 5 out of 7 mice died. These results may suggest the superiority of 2nd generation Ad-hHER3-FL viral vector above other Ad-HER3 vectors for vaccine use.

Establishing HER3 Transgenic Mice Colony:

We have acquired human HER3 transgenic mice (MMTV-rat neu/MMTV-human erbB3 transgenic) from Case Western Reserve (Dr. Stan Gerson). A pair of HER3 transgenic mice were imported from Case Western and are currently under breeding at our animal facility CCIF. They have been undergoing genotyping by PCR, and we have established a colony of human HER3 transgenic mice to confirm immunogenicity of the Ad-HER3 constructs.

For genotype testing, 0.3 cm tail clips were digested in proteinase K solution over night.

The human erb-B-3 primers were:

forward 5'-CCA TCT TCG TCA TGT TGA ACT ATA ACA C-3',

reverse: 5'-TAC ACA AAC TCC TCC ATA CTG ATA CTT-3'.

Thirty cycles of PCR were performed.

From one pair of HER3(+) parents, the colony of HER3 transgenic mice was successfully expanded and we currently have more than 200 HER3 transgenic mice ready for use in this study. Importantly, we have identified homozygous HER3 transgenic mice (3 males and 2 females), which will enable us to maintain transgenic mice colony without frequent genotyping.



Figure 13. Genotyping of HER3 Transgenic mice. Typical case of PCR genotyping for the offspring of HER3 transgenic parental mice from Case Western Reserve.

Immunogenicity Test in HER3 Transgenic Mice:

To confirm the findings of immunogenicity test of our new Ad-HER3 vectors performed in normal BALB/c

mice, we conducted the immunogenicity test of these vectors in HER3 Transgenic mice. IFN-gamma ELISPOT assay, Cell-based ELISA, flow-based assay were performed. Vaccinations were repeated with 2 weeks interval, and mice were sacrificed for immune assays a week after the boost vaccination.

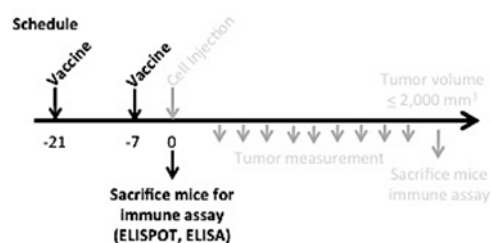


Figure 14. Scheme of Immunogenicity Testing in HER3 Transgenic Mice. On days -18 and -4, mice were vaccinated with Ad-vectors (2.6×10^{10} vp/mouse), and 4 mice from each group were sacrificed for immune assays on day 0.

Spleen was harvested for ELISPOT assay, and blood for test of antibody production.

a) Humoral Immune Response: We found established humoral immune responses in HER3 transgenic mice by flow-based assay as shown below in Figure 15. 4T1 (HER3 negative) or 4T1-HER3 (HER3 transfectant) cells were incubated with mouse sera, which were diluted with saline (1:100 dilution), then with PE-conjugated secondary antibody (anti-mouse IgG). Sera from Ad-GFP vaccinated mice were used as negative control, commercially available anti-HER3 mAb as positive control, and mouse serum from Ad[E1-]HER3 vaccinated mice were used for comparison purpose.

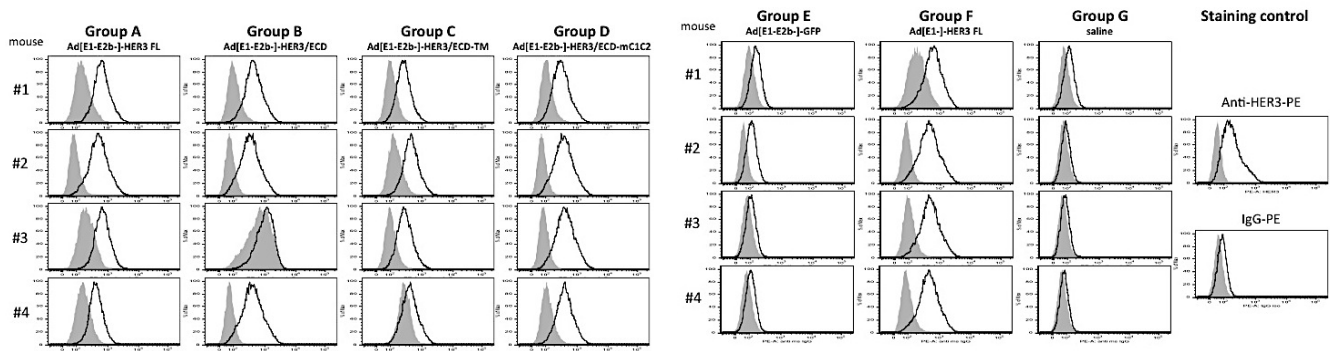


Figure 15. Anti-HER3 antibody levels in the serum of Ad-HER3 vaccinated HER3 transgenic mice. Mice were vaccinated with Ad-HER3-full length (FL), Ad-HER3/ECD, Ad-HER3/ECD-TM, Ad-HER3/ECD-C1C2, or control Ad-GFP, Ad[E1-]HER3 (2.6×10^{10} vp/vaccination). Two weeks later, vaccination was repeated with the same Ad vectors, and 7 days later, blood was collected to analyze the induction of anti-HER3 antibody. 4T1 cells (HER3-negative) and 4T1-HER3 cells (HER3 transfectant) were labeled with mouse sera (1:100 dilution) for 30 min, washed and then incubated with PE-conjugated 2ndary antibody (anti-mouse IgG) for 30 min. Open Histogram: 4T1-HER3 cells, Grey Histogram: 4T1 cells.

We further analyzed anti-HER3 antibody level in each serum by cell-based ELISA. 4T1 murine breast cancer cell line (HER3 negative) and human HER3 transfectant (4T1-HER3) were used in this assay. Sera from each mouse were titrated from 1:50 to 1:6400 (Figure 16).

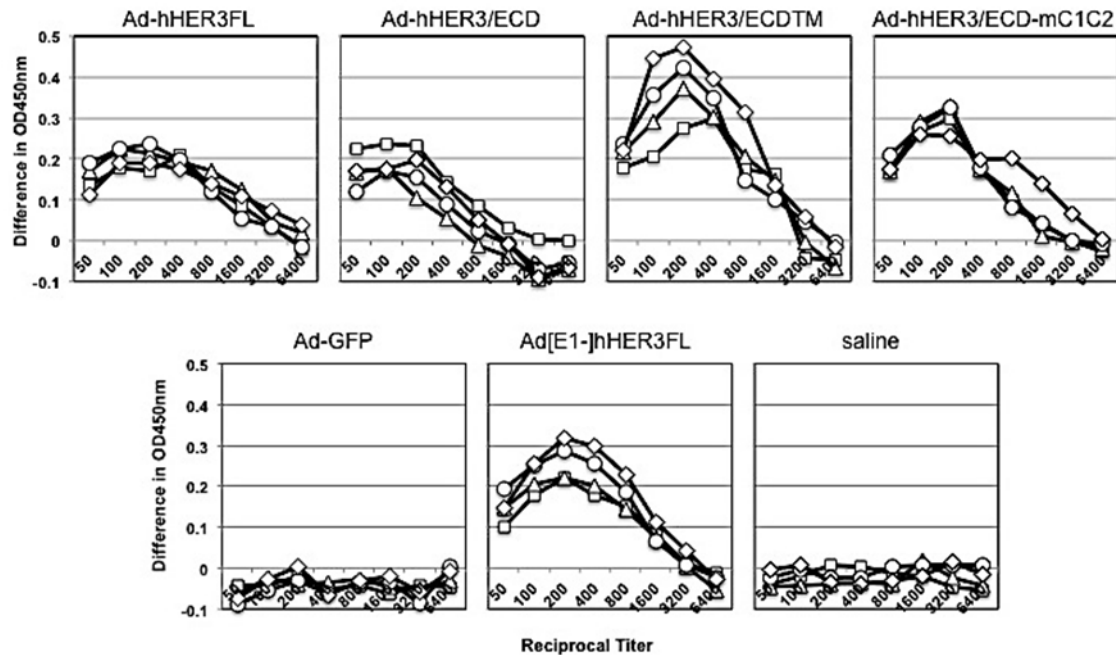


Figure 16. Cell-based ELISA with mouse serum from HER3 Transgenic Mice. HER3 Transgenic mice were vaccinated twice with Ad-HER3 vectors, Ad-GFP control or saline. Seven days after the last vaccine, mice were euthanized and serum was collected. 4T1 and 4T1-HER3 cells were seeded into 96 well plates. After overnight incubation, cells were washed with buffer, and mouse serum with serial dilutions were added (1:50 to 1:6400) and incubated for 1 h on ice. Then, cells were fixed, and HRP-labeled Goat anti-mouse IgG (1:2000) was added. After 1 h incubation, washed with PBS 3 times, and TMB was added for 5 min. Color development was stopped by adding H₂SO₄. Differences of OD₄₅₀ values (value for 4T1-HER3) – (value for 4T1) are shown for individual mice.

Among the four new Ad-HER3 vectors, Ad-HER3/ECD-TM induced the strongest humoral immune response against HER3, followed by Ad-HER3/ECD-C1C2, Ad-HER3-FL, and Ad-HER3/ECD.

b) Antigen-specific Cellular Response: We also analysed antigen-specific cellular immune response in HER3 Transgenic mice by IFN-gamma ELISPOT assay (Figure 17). As expected, only Adenoviral vectors encoding full length HER3 (Ad5(E2b-)HER3 FL and Ad(E1-)HER3 FL) induced T cell response for the intracellular domain of HER3 antigen. T cell responses against the peptide mix of HER3 extracellular domain were variable. Ad-HER3-FL, Ad-hHER3/ECD-TM, and Ad-HER3/ECD-TM induced similar levels of strong cellular response against extracellular domain of HER3. Ad(E1-)HER3 virus encoding full length, however, induced only weak cellular response against extracellular domain, probably because of neutralization by anti-Ad antibody induced by the priming vaccine.

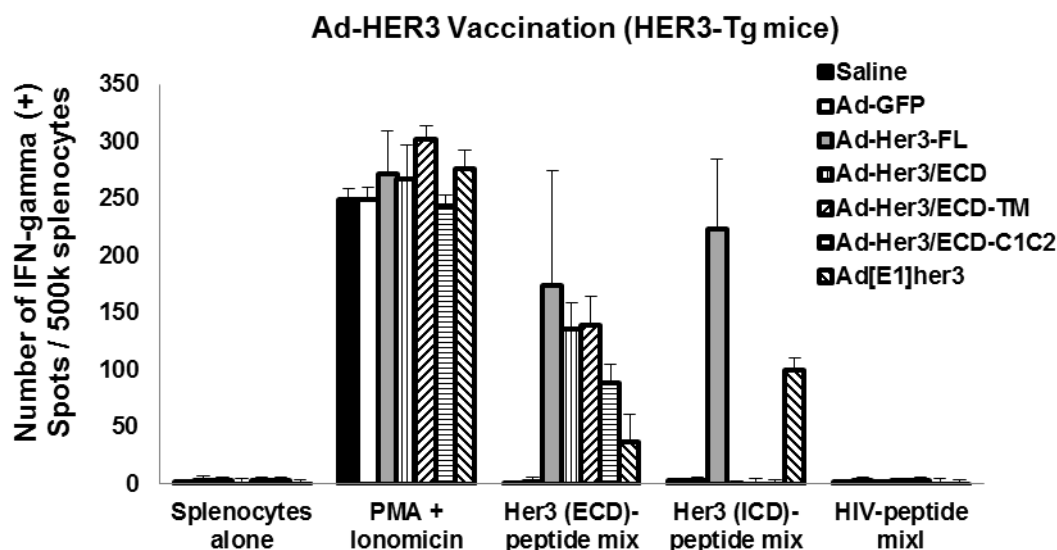


Figure 17. Anti-HER3 cellular response induced by Ad-HER3 vaccination in HER3 Transgenic Mice. HER3 Transgenic mice were vaccinated with Ad-HER3-full length (FL), Ad-HER3/ECD, Ad-HER3/ECD-TM, Ad-HER3/ECD-C1C2, or control Ad-GFP, Ad[E1]-HER3 (2.6×10^{10} vp/vaccination). Two weeks later, vaccination was repeated with the same Ad vectors, and 7 days later, spleen was collected to assess anti-HER3 cellular response. ELISPOT plates were coated with anti-IFN γ mAb overnight. 500K splenocytes were put into each well with HER3-ECD peptide pool, HER3-ICD peptide pool, HIV peptide pool (negative control) and PMA+Ionomycin (positive control). Cells were incubated overnight, and spots were developed. Average of 4 mice from each group are shown.

c) Antitumor Response: To assess the vaccine efficacy of newly made Ad-HER3 vectors, we plan to use HER3 transgenic mice model with implantation of human HER3-expressing murine breast cancer cells that derived from MMTV-neu mice (FVB background). We have established MNX5 murine breast cancer cell line from spontaneously occurring tumors in MMTV-neu female mice. We confirmed its tumorigenicity in MMTV-neu mice and HER3 Transgenic mice. Then, we generated MNX5-hHER3 cells using lentiviral vector encoding human HER3, with puromycin resistant gene as a selection marker. Unfortunately, MNX5-hHER3 cells grew in HER3 Transgenic mice for about 2 weeks, but eventually rejected by immune system.

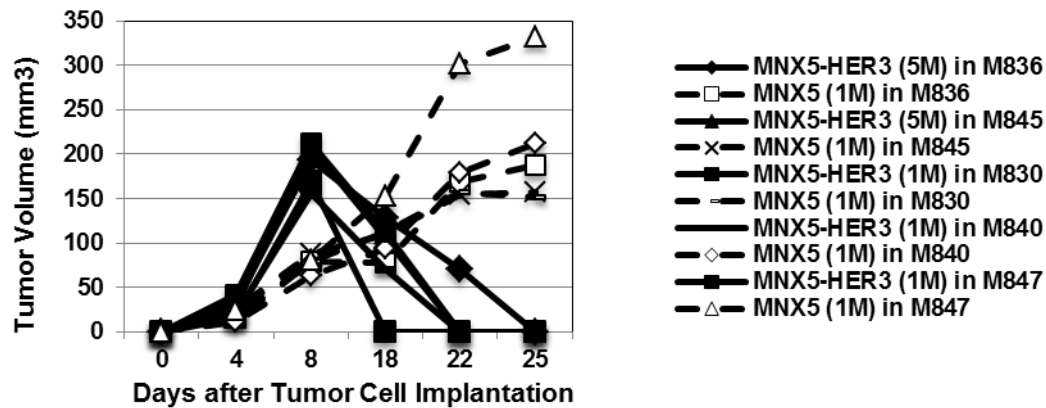


Figure 18. Tumorigenicity of MNX5-hHER3 cells and parental MNX5 cells in HER3 Transgenic mice. One million or 5 million tumor cells were resuspended in 50% Matrigel (100 μ L/injection), and subcutaneously injected into the flank of HER3 Transgenic mice. MNX5-HER3 tumor cells grew in first 10~14 days, however, was eventually rejected and disappeared by day 25. On the contrary, MNX5 parental cells grew steadily in HER3 transgenic mice. Similar trend was observed in MMTV-neu transgenic mice.

We hypothesized that puromycin-resistant gene, which is a foreign gene for mice, is inducing immune response in HER3 transgenic mice and leading to rejection of MNX5 HER3 transfected cells. Therefore, we started construction of new HER3 transfectant cells without any selection markers. To select HER3-positive cells from the whole MNX5 cell population, FACS sorting for HER3 expressing cells was repeated 3 times, and currently more than 95% of cells are HER3 positive (Figure 19). We are currently conducting tumorigenicity test with this newly generated MNX5-hHER3 cells in HER3 Transgenic mice.

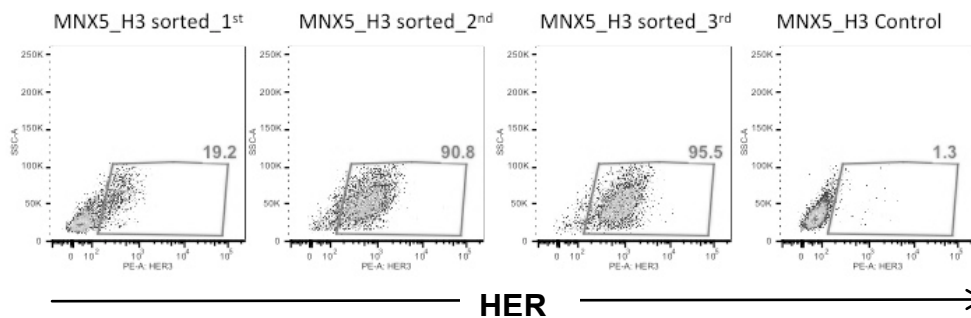


Figure 19. FACS sorting of HER3 positive MNX5 cells after transfection with lentiviral vector encoding human HER3 gene without selection markers. MNX5 breast cancer cells were transfected with lentiviral vector encoding human HER3 gene without selection markers. Cells were FACS sorted to isolate HER3 positive cells. After FACS sorting, HER3 positive MNX5-hHER3 cells were put back into culture and expanded in number. Sorting was repeated 3 times, and HER3 positivity reached 95.5%.

Once the tumorigenicity of MNX5-hHER3 cells in HER3 transgenic mice is confirmed, we will conduct the experiments to assess antitumor effect of Ad-HER3 vaccines with preventive model in HER3 transgenic mice.

Aim 1: Generation of GMP Ad5(E2b-)-HER3 (Y 1-3)**Task 1C: Generation of GMP Ad5(E2b-)-HER3 (Y1, Q3-4)**

Etubics has performed a due diligence site visit and meeting at Duke University. Dr. Frank Jones of Etubics has met with Dr. Kim Lyerly of Duke University who is the Principle Investigator (PI) on the project and his team. As a result of this meeting, project tasks have been assigned and agreed upon by Etubics and Duke University. Etubics has requested and received quotes for each task that has been assigned to the project for production and manufacture of the recombinant Ad5 [E1-, E2b-]-HER3 platform vector for clinical use.

Prior to performing a large-scale cGMP run for the manufacturing of the Ad5 [E1-, E2b-]-HER3 therapeutic product, the recombinant Ad5 [E1-, E2b-]-HER3 platform vector will be constructed and produced. A scale down GLP run at 5 Liter (5L) to produce Ad5 [E1-, E2b-]-HER3 will also be performed in order to conduct pre-clinical toxicity testing of the product and process developments.

Dr. Frank Jones and Elizabeth Gabitzsch, Vice President of Research, have performed a site visit at the CMO, SAFC, Carlsbad to plan and discuss the overall project specific objectives and timelines. Dr. Jones and Ms. Gabitzsch met with both the Process Development and GMP technical teams as well as the SAFC, Carlsbad site director. After discussions with the PD technical team a path forward has been agreed upon for the 5L scale down GLP run. This run will begin as soon Dr. Kim Lyerly of Duke gives approval as to the final HER3 target and as the vector has been produced and clearance testing has been performed. After the 5L Process Development (PD) run the product will be moved into the GMP suite for clinical material production applying the manufacturing parameters identified in the small scale GLP run. The details of the large scale GMP run have been agreed to by Etubics and SAFC's GMP team and the site director. Project timelines were reviewed and the executive team at SAFC has agreed that if the activities are initiated in a timely manner, December 2013, clinical grade material can be manufactured by Q4 2014. A manufacturing contract has been drawn up and negotiated and approved by Etubics and SAFC.

During the manufacturing process Etubics will complete documents and apply for RAC approval to use the Ad5 [E1-, E2b-]-HER3 product in human clinical trials. Etubics has previously achieved approval for its Ad5 [E1-, E2b-]-HER2 product candidate. This gives us confidence that we may achieve approval from the committee for the current Ad5 [E1-, E2b-]-HER3. Following RAC approval Etubics will initiate and hold a FDA Pre-IND meeting to ask all needed question and gain answers from the FDA to proceed to an IND. Once the Ad5 [E1-, E2b-]-HER3 product has been manufactured, tested, vialled and released, we will coordinate with the manufacturer to apply to the FDA for an IND to use the product in clinical trials.

Aim 1: Generation of GMP Ad5(E2b-)-HER3 (Y 1-3)**Task 1D: Development of a protein pathway signature of activated HER3 signaling (Y1-3)****Development of tamoxifen resistant models**

ER+ breast cancers are primarily treated with one of two broad classes of hormonal therapies. The first are the selective estrogen receptor modulators or SERMs. Tamoxifen (Tam) is the most widely used SERM, particularly in the treatment of ER+ breast cancer in pre-menopausal

women. SERMs target ER expressed in tumor cells. The second are aromatase inhibitors (AIs). The target of AIs, aromatase, is expressed in adipose and other normal tissues rather than breast cancer cells, making AIs extremely difficult to study in cell culture systems. Our first task therefore was to generate isotype matched pairs of ER+ human breast cancer cell lines: Tam-resistant cells and their treatment naive cell counterparts. Once having established these models, we would then determine differences in the cell signaling architecture that might help identify tumors that are more likely to respond to a HER3 vaccine strategy. Cell lines were selected based on the following two criteria. First, they had to be ER+ and sensitive to tamoxifen. And second, we selected ER+ breast cancer cell lines that expressed HER3 with variable expression of other HER receptor family members that form heterodimers with HER3 e.g. HER1/EGFR; HER2. We selected the cell lines based on previously published data from an article by Neve and colleagues who conducted a genomic and protein-based characterization of a panel of 49 breast cancer cell lines (3). In the article, the protein signal from Western blot was semi-quantitated using scanning densitometry. The ER and HER receptor protein profile of the four cell lines that we chose to develop Tam resistance are listed in Table 2.

Although our group has extensive experience developing models of resistance to targeted therapies e.g. HER2/EGFR tyrosine kinase inhibitors/TKIs, generating Tam-resistance proved to be particularly challenging. The delayed antitumor response to hormonal therapy made it difficult to adjust the tamoxifen dose to generate resistant cell lines. The sensitivity, as measured for tamoxifen by the ID20, a drug concentration resulting in 20% reduction in doubling time of the parental cell lines to tamoxifen was in the 10-500 nM range. After multiple attempts during a 4-6 month period, we were finally able to generate several Tam-resistant cell lines that maintain their viability despite growing in the continuous presence of $\geq 1 \mu\text{M}$ tamoxifen. In addition, we are in the process of developing an additional Tam-resistant cell lines, MDA-MB-134, which is ER positive (+++), EGFR (-), HER2 (+), HER3 (+++), and HER4 (-). Although it is not explicitly stated as a task, the Tam-resistant cell lines should be used to test the antitumor effects of antibodies generated by the HER3 vaccine, similar to our work evaluating the antitumor effects of anti-HER2 antibodies generated by a vaccine (4,5).

Table 2. Tamoxifen Resistant ER+ Breast Cancer Cell lines

Cell Line	ER	HER3	HER2	HER1 or EGFR	HER4
BT483	+++	++	-	-	-
MCF7	+++	+++	-	-	-
MDA-MB-361	+++	++	+++	+	-
T47D	+++	+++	+	-	+++

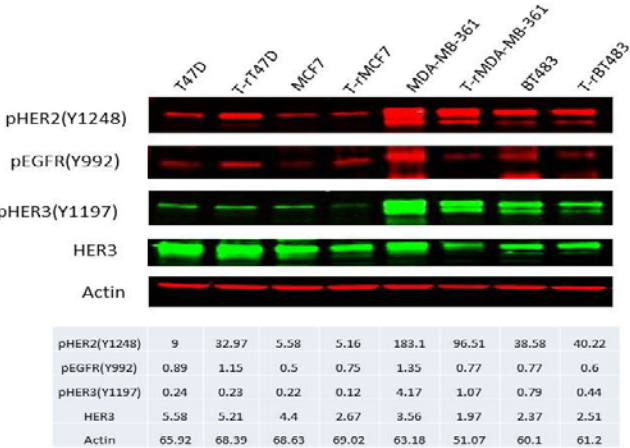
Comparison of signaling pathways in parental versus Tam-resistant cells.

We next addressed the question as to whether the expression of HER receptors (total and phosphorylated/activated forms) and downstream linked signaling pathways changed with the

development of Tam-resistance. We performed Western blot using primary antibodies against the indicated proteins and phosphoproteins according to methods that we have previously published (4,6). As shown, there was relatively little change in ER expression in Tam-resistance (e.g. T-rMCF7) versus parental cell counterparts (Figures 20A and B). Steady-state levels of HER3 and the activated tyrosine autophosphorylated form of HER3 (Y1187) were decreased in two of the Tam-

resistant cell lines (T-rMCF7; T-rMDA-MB-361) compared with parental controls.

A



B

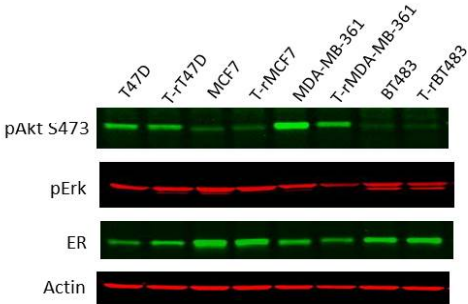


Figure 20. Comparison of Tam-resistant and parental controls. Western blot analysis of the indicated proteins/phosphoproteins as indicated in A and B. Actin steady-state protein levels served as a control to ensure for equal loading of protein. In (A), the absolute optical density (OD) values attributed to the bands- determined using the Odyssey Infrared Imaging System-are indicated under each corresponding lane. Results are representative of three independent experiments.

Demonstration of the functional role of HER3 in models of Tam-resistance.

We next sought to determine the functional role of HER3 in our models of Tam-resistance. Our initial studies compared the effects of targeted molecular knockdown of HER3 in Tam-resistant MCF7 (T-rMCF7) and T47D (T-rT47D) cell lines on the indicated protein/phosphoproteins (Figure 22). The siRNA knockdown of HER3, using previously published methods (6) was highly effective in both cells as documented by Western blot analysis (Figure 21, top panel).

Interestingly, the T-rMCF7 cells, but not T-rT47D, underwent apoptosis in response to HER3 knockdown as evidenced by increased expression of the Parp cleavage product (c-Parp) and inhibition of cell growth and viability (lower panel bar graphs).

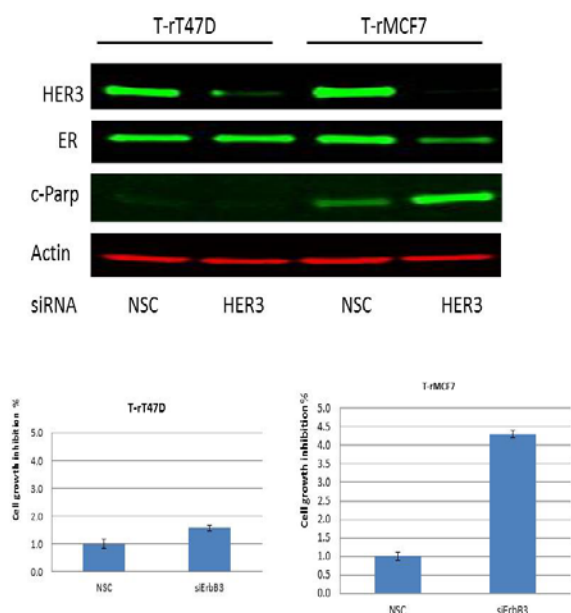


Figure 21. Differential effects of HER3 knockdown in Tam-resistant cells. (Top panel) Western blot analysis of the indicated proteins in T-rT47D and T-rMCF7 cells transfected with HER3 siRNA or scrambled siRNA control constructs (NSC). (Bottom panel) Effects of HER3 (ErbB3) siRNA mediated knockdown on T-r47D and T-rMCF7 cell growth and viability. Results represent the mean \pm standard error of triplicate samples, and are representative of three independent experiments.

These findings indicate that expression of HER3 alone does not predict for response to therapeutic approaches targeting HER3 e.g. anti HER3 antibodies generated as part of a HER3 vaccine. These findings are not dissimilar from previous observations made in HER2+ breast cancers, where overexpression of HER2 is not sufficient to predict for response to the anti-HER2 monoclonal antibody trastuzumab (Herceptin) (7). A deeper understanding of the tumor profile of ER+ breast cancers that are dependent on HER3 for cell survival is therefore required in order to identify patients more likely to respond to a HER3 targeted vaccine.

Identification of an activated HER3 signaling pathway.

HER3 is activated by its soluble ligand heregulin β 1 (HRG). Binding of HRG to HER3 triggers HER3 dimerization with a partnering HER receptor e.g. HER2, EGFR (8). In this regard, HER3-HER2 heterodimers are among the most potent activators of the anti-apoptosis PI3K-Akt-mTOR signaling pathway (9). The role of PI3K pathway signaling in the development of therapeutic resistance to cancer therapies is well established, so activators of this pathway are likely to promote therapeutic resistance. Heregulin has been shown to promote resistance to small molecule tyrosine kinase inhibitors (4,10) and trastuzumab (11). A recent study found that the HRG played a role in promoting resistance to the EGFR tyrosine kinase inhibitor (TKI) gefitinib in Tam-resistant MCF7 cells (12). Furthermore, the potential clinical relevance of these findings was demonstrated by showing that HRG was expressed in the cytoplasm of all 77 breast cancer samples analyzed from patients with locally advanced and metastatic breast cancers, 60% of which were ER+. In collaboration with the Lysterly group, we recently showed that autocrine production of HRG can mediate therapeutic resistance to lapatinib, a dual HER2 and EGFR TKI

approved for the treatment of HER2+ breast cancers (6). In lapatinib resistant breast cancer cells, the presence of HRG promoted a switch in the regulation of tumor cell survival from HER2-HER3-PI3K signaling in treatment naïve HER2+ breast cancer cells to an HRG-HER3-EGFR-PI3K signaling axis in resistant cells (6). We were able to demonstrate the persistent activation of an HRG-HER3 driven downstream signaling pathway that might help inform an activated HER3 protein pathway signature that is more likely to respond to the anti-tumor effects of a HER3 vaccine. In the BT474 luminal B breast cancer cell that overexpresses HER2, co-expresses HER3, and is ER+, we showed that HRG can abrogate the effects of a HER2 TKI (Figure 22). Details of the methods used for these experiments were recently published (6).

In addition, we used reverse phase phosphoprotein microarrays (RPMA)- a high throughput technology that can simultaneously quantitate the expression of hundreds of proteins/phosphoproteins involved in the major pathways that regulate tumor cell growth, cell cycle, DNA repair, and viability (13)- to characterize the effects of HRG in BT474 cells. We selected BT474 because it represents a cell that expresses ER, although it is primarily driven by HER2-HER3 dimers, and does not express HER4, the latter also a cognate receptor for HRG. Examples of the analysis are shown in Figure 23. As shown, some phosphoproteins change significantly in response to HRG, while others remain essentially unchanged. The changes induced by HRG, and magnitude of those changes are likely influenced by the repertoire of HER receptor expression, and the genomic profile of the tumor. For example, baseline phosphorylation of Akt on serine 473 (S473) is increased compared with phosphorylation of Erk (threonine 202/T202). We are now in a position to analyze the protein pathway architecture in our models of Tam-resistance in the presence or absence of HRG, using their treatment naïve cell counterparts as controls.

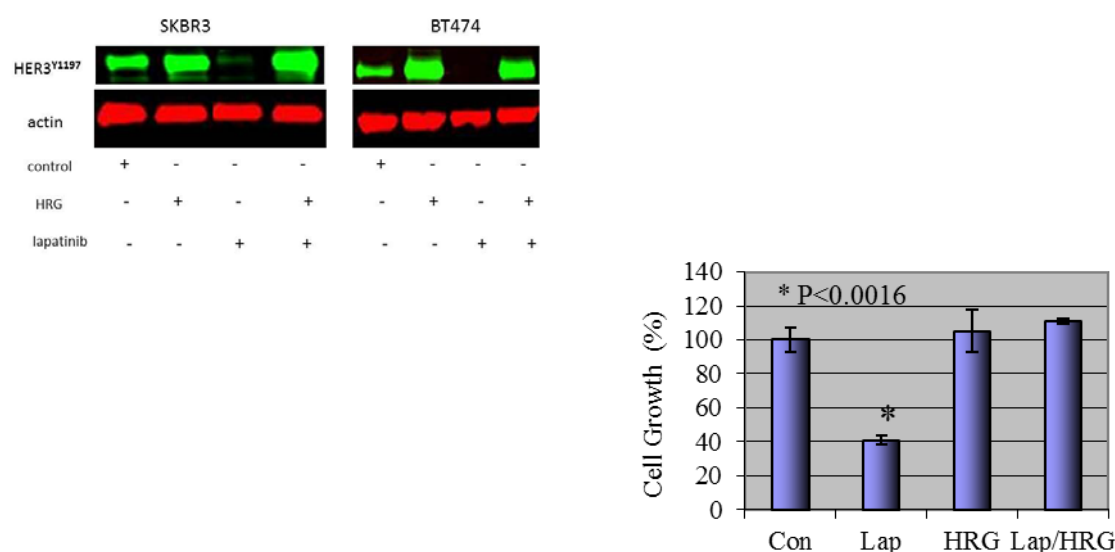


Figure 22. HRG activate HER3 and blocks BT474 cell sensitivity to lapatinib. Cells were treated for 48 hours with vehicle (DMSO), 1 μ M lapatinib, HRG (50 ng/ml), or the combination of 1 μ M lapatinib plus HRG (50ng/ml) in culture medium supplemented with 1% serum prior to analyzing their proliferative growth. Steady-state phospho-HER3 (Y1197) protein levels assessed by Western blot analysis using an HER3 phosphotyrosine specific antibody in response to the indicated treatment

conditions (Left Panel). Persistent HER3 signaling protects BT474 cells for lapatinib-induced apoptosis, $p < 0.0016$ (Right Panel). Results represent the mean \pm standard error of triplicate samples, and are representative of three independent experiments. The data was recently published (6).

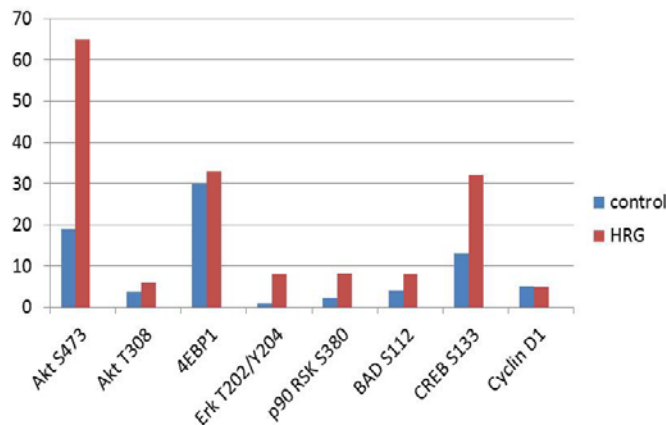


Figure 23. Effects of HRG-HER3 activation on signaling pathways. BT474 cells were treated with HRG (50ng/ml) for 24 hours before harvesting and analysis by RPMA. Results are representative of three independent experiments, each with triplicates samples per treatment condition. Untreated cells served as controls. The Y axis represents intensity values (x 10³).

Aim 2: Pre-clinical testing of activity and toxicity of Ad5(E2b-)HER3 (Y1-5)

Task 2A: Pre-IND meeting with FDA

Discussion with the FDA regarding a pre-IND meeting for the selected Ad5(E2b-)HER3 candidate vector and a pre-IND package for the Phase I clinical trial have been initiated.

Aim 2: Pre-clinical testing of activity and toxicity of Ad5(E2b-)HER3 (Y1-5)

Aim 2 Task 2D: Begin prospective tissue collection of tumors resistant to anti-estrogen therapy and explore expression of HER3 and the HER3 signaling pathway (Y1-5)

Tissue-based assay development

We have performed a series of immunohistochemical staining experiments using the anti-HER3 antibody C-17 from Santa Cruz. Using a number of cell line controls, we have observed predominantly cytoplasmic and some nuclear, but little membrane reactivity. The immunohistochemical staining pattern produced by the Santa Cruz antibody is not what we expected. It will be necessary to exclude nonspecific staining and to determine the performance characteristics of additional anti-HER3 antibodies. We have recently purchased a second anti-HER3 antibody that will be tested shortly. Recently we have obtained IRB and DOD approval of a protocol that allows us to study HER3 expression in human tissues.

Prospective tissue collection

Throughout the first year we have had thorough discussions both within this project and with other tissue procurement personnel at Duke about the best strategy for prospectively collecting tissues that can be used for biomarker assays. We have written a tissue procurement protocol and consent for IRB and DOD approval. Finally, we have devised a strategy to procure recurrent breast cancer tissue under image guidance.

3. KEY RESEARCH ACCOMPLISHMENTS

1. Generation of 4 different Ad5(E2b-)HER3 vectors, including Ad(E2b-)HER3 full length, Ad(E2b-)HER3/ECD, Ad(E2b-)HER3/ECD-TM and Ad5(E2b-)HER3/ECD-C1C2 constructs.
2. Confirmation of induced anti-HER3 cellular and humoral immune response by Ad-HER3 vaccine in mice (BALB/c).
3. Confirmation of antitumor effect of Ad-HER3 vaccination against HER3-expressing breast cancer cells in mice (JC-HER3 cells in BALB/c mice).
4. Establishment of HER3 Transgenic mouse colony in the Duke Cancer Center Isolation Facility (CCIF).
5. Confirmation of induced anti-HER3 cellular and humoral immune response by Ad-HER3 vaccine in HER3 transgenic mice.
6. Etubics has performed a due diligence site visit and meeting at Duke University.
7. Dr. Frank Jones and Elizabeth Gabitzsch, Vice President of Research of Etubics, have performed a site visit at the CMO, SAFC, Carlsbad to plan and discuss the overall project specific objectives and timelines for manufacture of Ad5 [E1-, E2b-]-HER3 product.
8. Establishment of multiple Tam-resistant ER+/HER3 expressing human breast cancer cell lines. These models will be used to identify the protein architecture associated with HER3 pathway activation.
9. Development of HER3 activated pathway signatures using heregulin (HRG) as an activator of the HER3 pathway.
10. Preliminary studies using HER3 targeted molecular knockdowns have identified HER3 expressing Tam-resistant cells that are dependent, and those that are not. Interrogating the protein signaling network in these models may inform the identification of an ER+/HER3 expressing tumor profile more likely to respond to a HER3 vaccine.
11. A tissue use protocol for breast tissue in Duke breast tissue banks has been approved by the Duke IRB and the DOD (eIRB# Pro000045010).

4. REPORTABLE OUTCOMES:

1. Development of new generation Ad5(E2b-)HER3 vectors
2. Generation of mouse model for HER3 targeting (JC-HER3 cells in BALB/c mice, MNX5-HER3 cells in MMTV-neu/MMTV-hHER3 transgenic mice)
3. Development of HER3 transfectant murine breast cancer cell lines (JC-HER3, MNX5-HER3)
4. Development of multiple isogenic pairs of parental and Tam-resistant human ER+ breast cancer cell lines.
5. Manuscript by Xia et al. Breast Cancer Research 2013, 15:R85 (see Appendix 1).
6. Manuscript by Morse et al. Morse et al. Cancer Immunol Immunother 2013, 62:1293-130 (see Appendix 2).

5. CONCLUSION:

The preclinical immunogenicity and tumorigenicity testing of four Ad5(E2b-)HER3 constructs are ongoing in a HER3 transgenic mouse model (rat neu/human HER3/FVB strain). We will advance the most immunogenic construct and meet with the FDA to discuss IND submission for GMP grade candidate.

HER3 has been implicated as playing a role in the development of resistance to hormonal therapies used in the treatment of ER+ breast cancer. In an attempt to identify patients who are more likely to respond to a HER3 vaccine, our objective is to develop a tumor signature associated with an activated HER3 signaling pathway. Our working hypothesis is that ER+ breast cancers that are driven by HER3 are more likely to be sensitive to the effects of a HER3 vaccine. To test this hypothesis, we first needed to generate Tam-resistant models that expressed HER3, which we have done over the past year. Interestingly, total HER3 protein expression was not increased in Tam-resistant cells compared with treatment naïve cell counterparts. In fact, HER3 protein appears to be decreased in two of the resistant cell lines compared to controls. Moreover, in molecular knockdown studies, we showed that HER3 expression alone did not correlate with dependence upon HER3 signaling for cell survival. Other factors are clearly involved. We have begun to develop a protein pathway map associated with ligand (HRG) induced activation of HER3 signaling using the reverse phase phosphoprotein microarray technology. Once developed, the HRG-HER3 signature will serve as a reference point when we perform RPMA analysis on the Tam-resistant cell lines. It will be interesting to see whether the Tam-resistant cell lines that are sensitive to HER3 knockdown, express a protein pathway signature associated with activated HER3 signaling. Furthermore, we will examine the antitumor effects of antibodies generated by the HER3 vaccine in animals on signaling pathways and cell viability in Tam-resistant cell lines.

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7. APPENDICE:

Appendix 1: Morse et al. *Cancer Immunol Immunother* 2013, 62:1293-1301

Novel adenoviral vector induces T-cell responses despite anti-adenoviral neutralizing antibodies in colorectal cancer patients

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Abstract First-generation, E1-deleted adenovirus subtype 5 (Ad5)-based vectors, although promising platforms for use as cancer vaccines, are impeded in activity by naturally occurring or induced Ad-specific neutralizing antibodies. Ad5-based vectors with deletions of the E1 and the E2b regions (Ad5 [E1-, E2b-]), the latter encoding the DNA polymerase and the pre-terminal protein, by virtue of diminished late phase viral protein expression, were hypothesized to avoid immunological clearance and induce more potent immune responses against the encoded tumor antigen transgene in Ad-immune hosts. Indeed, multiple homologous immunizations with Ad5 [E1-, E2b-]-CEA(6D), encoding the tumor antigen carcinoembryonic antigen (CEA), induced CEA-specific cell-mediated immune (CMI) responses with antitumor activity in mice despite the presence of preexisting or induced Ad5-neutralizing antibody. In the present phase I/II study, cohorts

of patients with advanced colorectal cancer were immunized with escalating doses of Ad5 [E1-, E2b-]-CEA(6D). CEA-specific CMI responses were observed despite the presence of preexisting Ad5 immunity in a majority (61.3 %) of patients. Importantly, there was minimal toxicity, and overall patient survival (48 % at 12 months) was similar regardless of preexisting Ad5 neutralizing antibody titers. The results demonstrate that, in cancer patients, the novel Ad5 [E1-, E2b-] gene delivery platform generates significant CMI responses to the tumor antigen CEA in the setting of both naturally acquired and immunization-induced Ad5-specific immunity.

Keywords Immunotherapy · Ad5 vector · CEA · Cell-mediated immunity

Electronic supplementary material The online version of this article (doi:10.1007/s00262-013-1400-3) contains supplementary material, which is available to authorized users.

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Introduction

Cancer immunotherapy achieved by delivering tumor-associated antigens (TAA) has recently demonstrated survival benefits [1, 2]; however, limitations to these strategies exist and more immunologically potent vaccines are needed. To address the low immunogenicity of self-tumor antigens, a variety of advanced, multi-component vaccination strategies including co-administration of adjuvants and immune-stimulating cytokines have been employed [3, 4]. Alternatives include the use of recombinant viral vectors that inherently provide innate pro-inflammatory signals while simultaneously engineered to express the antigen of interest. Of particular interest are adenovirus serotype-5 (Ad5)-based immunotherapeutics that have been repeatedly used in humans to induce robust T-cell-mediated immune (CMI) responses all while maintaining an extensive safety profile [5–7]. In addition, Ad5 vectors can be reliably manufactured in large quantities and are stable for storage and delivery for outpatient administration [6–8]. Nonetheless, a major obstacle to the use of first-generation (E1-deleted) Ad5-based vectors is the high frequency of preexisting anti-adenovirus type 5 neutralizing antibodies. These antibodies can be present in a potential vaccinee due to either prior wild-type adenovirus infection [8, 9] or induction of adenovirus neutralizing antibodies by repeated injections with Ad5-based vaccines, each resulting in inadequate immune stimulation against the target TAA [10].

Attempts to overcome anti-Ad immunity have included use of alternative Ad serotypes and/or alternations in the Ad5 viral capsid protein, each with limited success and the potential for significantly altering biodistribution of the resultant vaccines. Therefore, a completely novel approach was attempted by further reducing the expression of viral proteins from the E1-deleted Ad5 vectors, proteins known to be targets of preexisting Ad immunity. Specifically, a novel recombinant Ad5 platform has been described with deletions in the early 1 (E1) gene region and additional deletions in the early 2b (E2b) gene region (Ad5 [E1-, E2b-]) [11]. Deletion of the E2b region (that encodes DNA polymerase and the pre-terminal protein) results in decreased viral DNA replication and late phase viral protein expression. This vector platform has been previously reported to successfully induce CMI responses in animal models of cancer and infectious disease [10, 12–18], and more importantly, this recombinant Ad5 gene delivery platform overcomes the barrier of Ad5 immunity and can be used in the setting of preexisting and/or vector-induced Ad immunity [10, 12–19], thus enabling multiple homologous administrations of the vaccine. We have constructed and tested an Ad5 [E1-, E2b-] platform containing a gene insert for the tumor antigen carcinoembryonic antigen (CEA) with a modification that enhances T-cell responses (Ad5 [E1-,

E2b-]-CEA(6D) [12, 16, 19, 20]. Multiple immunizations with this Ad5 platform induced CEA-specific CMI responses with antitumor activity despite the presence of existing Ad5 immunity in mice [12, 16]. We now present results of a first-in-man, phase I/II clinical trial to determine the safety and immunogenicity of dose escalation of the Ad5 [E1-, E2b-]-CEA(6D) vector in advanced stage colorectal cancer patients to determine whether CMI could be induced and whether there was an effect on clinical outcome relative to the existence of preexisting Ad5 immunity.

Methods

Construction and production of Ad5 [E1-, E2b-]-CEA(6D)

The cDNA sequence containing the modified CEA with the CAPI(6D) mutation was produced at Duke University [21]. Clinical grade Ad5 [E1-, E2b-]-CEA(6D) was constructed as previously described [12] and manufactured using the E.C7 cell line [12] under GMP at SAFC, Carlsbad, California, and provided by Etubics Corporation.

Protocol schema and patient treatment

The clinical study was performed under an FDA-approved Investigational New Drug Exemption (IND14325) and registered at ClinicalTrials.gov (NCT01147965). Participants were recruited from medical oncology clinics at Duke University Medical Center, Durham, NC, and Medical Oncology Associates, Spokane, WA. Patients provided informed consent approved by the respective Institutional Review Boards (IRB). Eligibility requirements included metastatic cancer expressing CEA and adequate hematologic, renal, and hepatic function. Trial participants were required to have received treatment with standard therapy known to have a possible overall survival benefit or refused such therapy. Exclusion criteria included chemotherapy or radiation within the prior 4 weeks, history of autoimmune disease, viral hepatitis, HIV, or use of immunosuppressives. Patients who had been receiving bevacizumab or cetuximab for at least 3 months prior to enrollment were permitted to continue receiving these antibodies. Prior CEA immunotherapy was permitted. The study employed a standard 3 + 3 dose escalation strategy with dose-limiting toxicities (DLT) defined as grade 3 or 4 major organ toxicity. The Ad5 [E1-, E2b-]-CEA(6D) doses were delivered to patients as follows: cohort 1: dose of 1×10^9 VP in 0.5 ml subcutaneously (SQ) in the same thigh every 3 weeks for 3 treatments; cohort 2: dose of 1×10^{10} VP in 0.5 ml SQ every 3 weeks for 3 treatments; cohort 3: dose of 1×10^{11} in 0.5 ml SQ every 3 weeks for 3 treatments.

Following the establishment of the dose of 1×10^{11} VP as safe, an additional 12 patients received Ad5 [E1-, E2b-]-CEA(6D) at this dose and schedule (phase II cohort). After completing the phase II cohort, an additional cohort (cohort 5) of six patients received a dose of 5×10^{11} VP in 2.5 ml SQ every 3 weeks for 3 treatments to determine safety of the highest achievable dose. PBMCs were collected from patients just prior to the immunizations at weeks 0, 3, 6, and three weeks following the last treatment. The PBMCs were frozen in liquid nitrogen until ELISPOT assays were performed. In cohort 5, fresh PBMCs were analyzed in preliminary flow cytometry assays for polyfunctional CD8+ T lymphocytes.

Assessment of clinical activity

Clinical activity was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST 1.0 criteria [22]) using computed tomography (CT) or magnetic resonance imaging (MRI) scans obtained at baseline and after treatments were completed. Toxicity was assessed according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 [23]. Peripheral blood CEA levels, hematology, serum chemistries, and anti-nuclear antibody titers were compared at baseline and 3 weeks following the final treatment. Survival was measured from the day of the first immunization until death from any cause.

Analysis of CMI responses by ELISPOT assay

An ELISPOT assay for IFN- γ -secreting lymphocytes was adapted from our previous animal studies and performed as described [12]. Briefly, isolated PBMCs (2×10^5 cells/well) from individual patient samples were incubated 36–40 h with a CEA peptide pool (15mers with 11aa overlap covering full-length CEA with the 6D modification; 0.1 μ g/well) to stimulate IFN- γ -producing T cells. CMI responses to Ad5 were determined after exposure of patient PBMC to Ad5 null (empty vector). Cells stimulated with concanavalin A (Con A) at a concentration of 0.25 μ g/well served as positive controls. Colored spot-forming cells (SFC) were counted using an Immunospot ELISPOT plate reader (Cellular Technology, Shaker Heights, OH), and responses were considered to be positive if 50 SFC were detected/ 10^6 cells after subtraction of the negative control and SFC were \geq twofold higher than those in the negative control wells.

Determination of Ad5 neutralizing antibody (NAb) titers

Endpoint Ad5 NAb titers were determined as previously described [12–14]. Briefly, dilutions of heat-inactivated test sera in 100 μ L of DMEM containing 10 % fetal calf

serum were mixed with 4×10^7 VP of Ad5 [E1-]-null and incubated for 60 min at room temperature. The samples were added to microwells containing HEK293 cells cultured in DMEM containing 10 % heat-inactivated calf serum at 2×10^3 cells/well for 24 h at 37 °C in 5 % CO₂. The mixture was incubated for an additional 72 h at 37 °C in 5 % CO₂. An MTS tetrazolium bioreduction assay (Promega Corp. Madison, WI) [24] was used to measure cell killing and endpoint Ad5 NAb titers. Endpoint titers with a value less than 1:25 were assigned a value of 0.

Statistics

Statistical analyses comparing immune responses were performed employing the Mann–Whitney test (PRISM, GraphPad). Survival comparisons were made employing Kaplan–Meier plots (PRISM, GraphPad). Ad5 NAb titer and CEA-specific CMI were analyzed as continuous variables. The association of Ad5 NAb titer with change in CEA-specific CMI was tested with the Spearman correlation coefficient. The association of Ad5 NAb titer with survival was tested with the Wald test of the proportional hazards model. All tests used a two-sided alpha of 0.05.

Results

Patient demographics and safety and tolerability

Thirty-two patients with metastatic colorectal cancer, median age 57.5 (range 38–77) who had failed a median of three prior chemotherapeutic regimens (range 2–5), had a performance status of 90 % (range 70–100 %), and had three sites of metastatic disease (range 1–4), were enrolled (Table 1). The majority were able to receive all three immunizations. All four patients who stopped immunizations early did so due to significant disease progression. There was no dose-limiting toxicity and no serious adverse events (SAE) that resulted in treatment discontinuation at any vaccine dose level. The most common toxicity (see Supplemental Table 1) was a self-limited, injection site reaction. Other reactions occurred with less than a 10 % incidence and included fever, flu-like symptoms, anorexia, chills, nausea, and headache. These symptoms were also self-limiting and did not require intervention other than symptomatic measures such as acetaminophen. Routine hematology and chemistry studies showed no significant biologic changes during the immunization period (Supplemental Table 2). In particular, the total lymphocyte count remained stable (pre and post). Overall, comparisons of ANA titers at baseline and 3 weeks after the last immunization revealed no significant difference in values across all patient groups (Supplemental Table 2).

Table 1 Patient demographics

Patient ID/cohort	Dose (VP)	Dx	Age	Sex	KPS	# prior CTx	Mets (# of sites)	# of doses	++Disease Status after tx	Survival (Months)
002/1	10 ⁹	C	67	M	70	>3	4	3	PD	3 (–)
003/1	10 ⁹	R	63	M	100	5	2	3	PD	9 (–)
004/1	10 ⁹	C	53	F	100	2	3	3	PD	11 (–)
005/2^	10 ¹⁰	C	60	M	100	3	3	3	SD	12 (+)
007/2	10 ¹⁰	C	52	M	80	2	5	1	PD	1 (–)
008/2	10 ¹⁰	C	42	F	100	3	3	3	PD	12 (+)
010/2	10 ¹⁰	C	58	M	90	3	3	3	PD	12 (–)
011/3	10 ¹¹	R	50	M	100	5	1	3	PD	12 (+)
012/3	10 ¹¹	C	48	M	100	1	2	3	PD	12 (+)
013/3	10 ¹¹	R	62	M	100	3	2	3	PD	4 (–)
500/3	10 ¹¹	C	55	M	80	4	3	3	PD	12 (+)
015/3	10 ¹¹	C	58	F	80	3	4	3	PD	10 (–)
016/3@	10 ¹¹	C	53	F	100	3	4	3	PD	6 (–)
017/3*	10 ¹¹	R	52	F	90	3	2	3	PD	3 (–)
501/II	10 ¹¹	R	54	M	90	1	1	3	PD	12 (+)
502/II	10 ¹¹	C	66	F	80	1	2	2	PD	3 (–)
019/II	10 ¹¹	C	69	M	90	1	3	3	PD	12 (+)
020/II^	10 ¹¹	C	59	M	100	5	4	3	SD	12 (+)
021/II^	10 ¹¹	C	51	F	100	4	3	3	PD	12 (+)
506/II	10 ¹¹	C	77	F	80	2	2	3	PD	3 (–)
023/II	10 ¹¹	C	51	F	100	3	4	3	PD	4 (–)
504/II	10 ¹¹	C	57	M	90	3	3	3	PD	12 (+)
507/II	10 ¹¹	R	58	M	90	2	2	3	PD	12 (+)
024/II	10 ¹¹	C	67	M	90	2	3	3	PD	12 (+)
025/II	10 ¹¹	C	62	F	100	2	4	3	PD	7 (–)
026/II	10 ¹¹	C	53	M	100	3	2	2	PD	4 (–)
030/5	5 × 10 ¹¹	C	38	M	90	4	3	3	PD	10 (+)
031/5	5 × 10 ¹¹	R	72	F	90	4	2	3	SD	9 (+)
032/5@	5 × 10 ¹¹	R	53	M	90	4	3	3	PD	6 (–)
033/5	5 × 10 ¹¹	R	48	F	90	>3	2	3	PD	5 (–)
034/5	5 × 10 ¹¹	C	62	M	100	5	4	3	PD	7 (+)
035/5	5 × 10 ¹¹	C	60	F	90	3	5	2	PD	2 (–)

Dx diagnosis, C colon, R rectal cancer, KPS Karnofsky performance status, PD progressive disease, SD stable disease

* concurrent cetuximab; ^concurrent bevacizumab; @ concurrent panitumumab

++Represents disease status at 9 weeks post-initiation of immunizations

(+) Alive; (–) Dead at last follow-up

Determination of Induced CMI Responses to CEA

ELISPOT analysis was performed on cryopreserved PBMC samples drawn before each immunization and after the completion of the final immunization to assess CEA-specific CMI responses. We observed a dose–response effect with the highest magnitude CEA-specific CMI responses occurring in patients who received the highest dose of Ad5 [E1-, E2b-]-CEA(6D) (Fig. 1). Of the doses received, 0/3 (0 %) patients in cohort 1 exhibited positive CEA-directed CMI responses, 1/4 (25 %) patient in cohort 2 exhibited

positive CEA-directed CMI responses, 10/19 (53 %) patients in cohort 3/phase II exhibited positive CEA-directed CMI responses, and 4/6 (67 %) patients in cohort 5 exhibited positive CEA-directed CMI responses. The time course of induction of CEA-specific CMI (Supplemental Fig. 1) demonstrated that there may be plateau in the magnitude of CEA CMI prior to the last dose although small numbers could affect this finding. In the largest group of patients who received the same dose (cohort 3 plus phase II), we observed a significant increase over baseline in the average CEA-directed CMI responses at the week 6

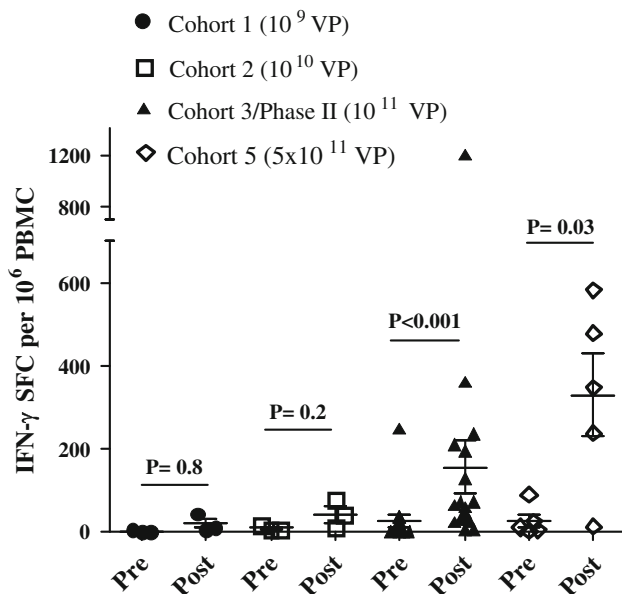


Fig. 1 CEA-directed CMI responses in treated patients. CMI (IFN- γ secretion) was assessed at baseline (pre) and after administrations of Ad5 [E1-, E2b-]-CEA(6D) (post). The highest CMI responses (regardless of time point) observed in the patients after treatment revealed a dose response. The highest CMI levels occurred in patients that received the highest dose of 5×10^{11} VP (Cohort 5). The CMI responses for cohort 3/phase II and cohort 5 were significantly elevated (*Mann–Whitney test*) as compared to their baseline (pre) values. Specificity of the responses was demonstrated by the lack of reactivity with the irrelevant antigens β -galactosidase and HIV-gag (data not shown). For positive controls, PBMCs were exposed to concanavalin A (data not shown). Horizontal line and error bar indicate the mean \pm SEM for each cohort

evaluation ($P < 0.05$, *Mann–Whitney test*), averaging 94 SFC/ 10^6 PBMC, which increased further by the week 9 evaluation (Supplementary Fig. 1). One patient (patient ID 13) had a highly elevated baseline CEA-specific immune response (1100 SFC) and had elevated CMI at week six (2305 SFC) but did not return for week 9 evaluation and therefore was not included in CEA CMI data analysis.

We also measured Ad5 NAb and CMI against Ad5 and correlated it with CEA-specific CMI. Each patient had their serum and PBMC sample tested at baseline (prior to treatment) and at 9 weeks after completion of 3 treatments. Nineteen of 31 patients (61.3 %) tested in this study had Ad5 neutralizing activity in serum samples prior to the onset of treatment with the CEA(6D)-expressing Ad vaccine. The mean pre-treatment Ad5 NAb titer value obtained among all patients was $1:189 \pm 1:71$ SEM (geometric mean 1:21), and the mean pre-treatment Ad5 NAb titer among seropositive patients was $1:308 \pm 1:108$ (geometric mean 1:146). Analysis of serum samples from patients who received 3 immunizations revealed Ad5 NAb titers that were significantly increased ($P < 0.0001$, *Mann–Whitney test*) by week 9 (mean $1:4767 \pm 1:1225$ SEM) (geometric mean 1:1541) when compared with their respective baseline values (Fig. 2a). Analysis of PBMC for CMI responses to Ad5 also revealed a significant increase ($P < 0.01$, *Mann–Whitney test*) in Ad5-directed CMI responses after immunizations with Ad5 [E1-, E2b-]-CEA(6D) (Fig. 2b). Only ELISPOT assays were performed for CMI, and we did not assess the relative contribution of CD4+ and CD8+ T cells; thus, it is unclear whether both cell types are responding or whether responses are associated preferentially from one group.

Comparison of week 9 CEA-directed CMI responses from patients with low baseline preexisting Ad5 immunity (Ad5 NAb ≥ 200) versus those with high baseline Ad5 immunity (Ad5 NAb > 200) revealed no significant difference in responses ($P > 0.4$, *Mann–Whitney test*). Further, when the highest CEA-specific CMI responses were compared with preexisting or vector-induced Ad5 NAb activity, there was no correlation between levels of CEA CMI and Ad5 NAb activity (Fig. 3). These data indicate that immunizations with Ad5 [E1-, E2b-]-CEA(6D) were able to induce CEA-specific immune responses in colorectal cancer patients despite the presence of existing and/or immunization-induced Ad5 immunity.

Fig. 2 Ad5 immune responses. Ad5 NAb titers **a** and CMI responses **b** to Ad5 were determined in patients at baseline (week 0) and 3 weeks (week 9) after the third immunization. The number of IFN- γ -secreting PBMCs from patients that were specific for Ad5 was determined by ELISPOT. Both the Ad5 NAb titers and Ad5 CMI responses were significantly elevated at week 9 (*Mann–Whitney test*). Horizontal line and error bar indicate the mean \pm SEM

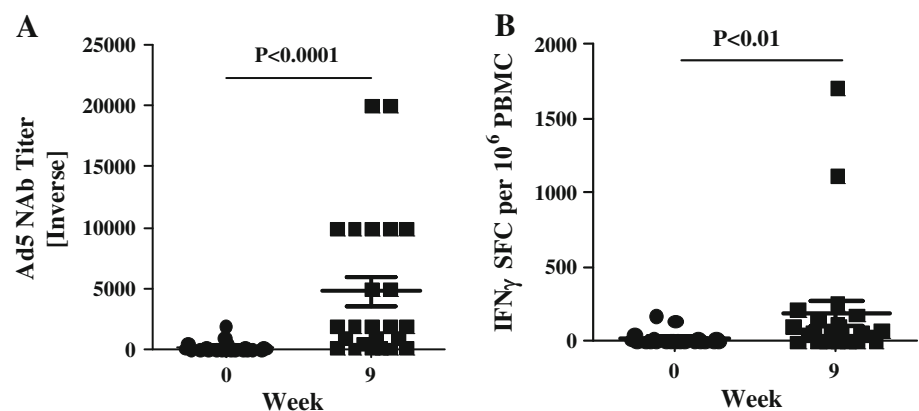
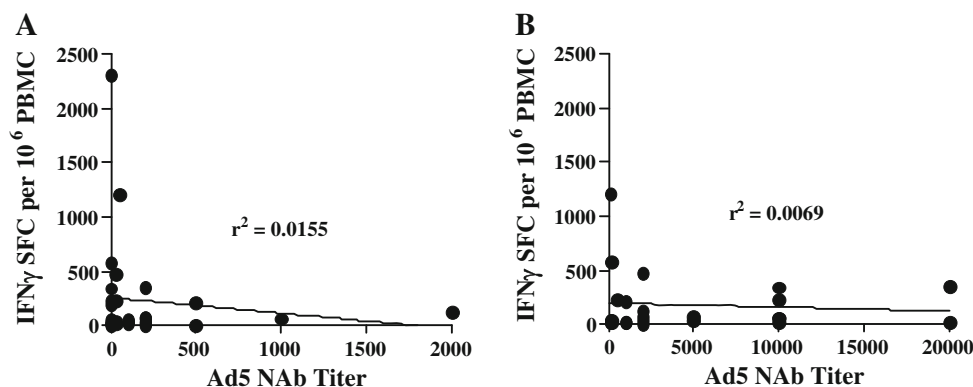


Fig. 3 CEA-specific immunity in patients and comparisons with Ad5 immunity. Correlation between preexisting Ad5 NAb activity and highest levels of induced CEA CMI responses **a.** Correlation between vector-induced Ad5 NAb activity and CEA CMI responses **b.** The r^2 values revealed no correlation between preexisting or vector-induced Ad5 NAb activity and CEA CMI ELISPOT responses



Clinical outcomes

Carcinoembryonic antigen levels in serum at baseline and week 9 were assessed in patients. Among those with CEA levels available at baseline and follow-up, three had no increase in CEA levels at the end of the immunization period while the remaining patients showed increased CEA levels. There were three patients with stable disease who remained so during the 9-week study period. All other patients experienced some level of progressive disease (Table 1). Patients in cohorts 1, 2, 3, and phase II who received at least 2 treatments ($n = 25$) were followed for survival and Kaplan–Meier plots and survival probabilities performed. Patients in cohort 5 ($n = 6$) have not completed the 12-month follow-up period and, therefore, were not evaluated for survival by Kaplan–Meier plots. Six patients in cohorts 1 and 2 experienced a 12-month survival probability of 33.3 % (Fig. 4). Nineteen patients in the combined group of cohort 3 and phase II experienced a 12-month survival probability of 52.6 % (Fig. 4). With a median follow-up of 12 months, all 25 patients as a group (cohorts 1, 2, 3, and phase II) experienced a 12-month

survival probability of 48 % (Fig. 4). There was no association between Ad5 NAb and survival using Ad5 NAb both as a continuous variable and as a variable dichotomized between <200 and ≥ 200 (P values 0.48 and 0.44, respectively). These data indicate that preexisting Ad5 NAb did not significantly impact survival outcomes following immunization with the Ad5 [E1-, E2b-]-CEA(6D) vaccine.

Discussion

Adenoviral vectors have significant potential for use as cancer therapeutic vaccines because of their propensity to induce robust adaptive immune responses specifically against transgene products in general; however, recombinant first-generation Ad5 [E1-] vectors used in homologous prime/boost regimens have been greatly limited in their potential efficacy due to the presence of preexisting Ad5 immunity as well as vector-induced immunity [7–10]. Specifically, Ad5-directed immunity mitigates immune responses to TAA that have been incorporated into earlier

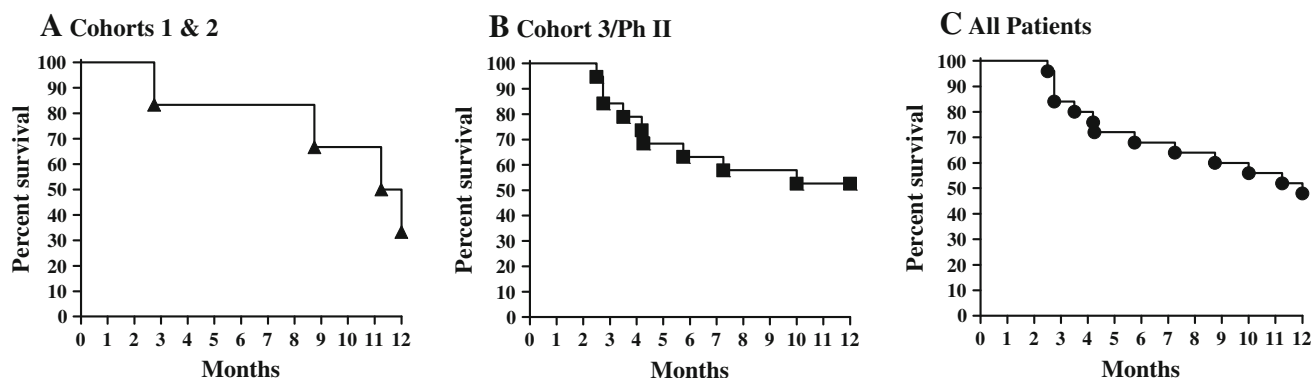


Fig. 4 Kaplan–Meier survival plots of patients treated with Ad5 [E1-, E2b-]-CEA(6D). Patients treated at least two times with Ad5 [E1-, E2b-]-CEA(6D) were followed for survival. *Panel a* represents 6 patients in cohorts 1 and 2 that were followed for survival. There were

4 events in this group. *Panel b* represents 19 patients in cohort 3 and phase II that were followed for survival. There were 9 events in this group. *Panel c* represents all 25 patients (cohorts 1, 2, 3, and phase II) that were followed for survival. There were 13 events in this group

generation Ad5 [E1-]-based platforms [10]. The Ad5 [E1-, E2b-] platform utilized in the present study was intended to accommodate a homologous prime–boost regimen, by avoiding presentation of antigens that are the targets of preexisting Ad5 immunity [2, 8, 25–28]. Since CEA has been identified as one of the priority cancer antigens by the National Cancer Institute [29], we investigated this TAA as a transgene to be incorporated into the new Ad5 [E1-, E2b-] vector platform for use as a cancer therapeutic vaccine. CEA expression in adults is normally limited to low levels in the gastrointestinal epithelium, whereas CEA is over-expressed in adenocarcinomas of the colon and rectum and in many breast, lung, and pancreas cancers [30, 31]. We chose the HLA A2-restricted CAP1(6D) modification of CEA because, compared with the wild-type CAP1 epitope, CAP1(6D) has been shown to enhance the sensitization of CTLs [19, 20] and has been included in our recent CEA-based vaccine constructs [32, 33]. Although we did not test for HLA type because we used full-length CEA that is not HLA-restricted, A*0201 is the allele observed most frequently in Caucasians (allele frequency 0.2717) and is common in other populations [34]. However, in expanded trials, we plan to test patients for HLA type and assess whether or not there may be a relationship between HLA type and clinical and/or CMI responses.

Previously, we tested multiple subcutaneous immunizations employing three administrations of a single dose level (1×10^{10} VP) of this class of Ad5 vaccine expressing the TAA CEA, (Ad5 [E1-, E2b-]-CEA(6D)) in a preclinical murine model of CEA-expressing cancer. In mice with preexisting Ad5 immunity, we demonstrated the induction of potent CEA-directed CMI responses that resulted in anti-tumor activity and noted that these CMI and anti-tumor responses were significantly greater than those responses induced by a current generation Ad5 [E1-]-based vector vaccine [12, 16]. We have also demonstrated in additional animal models (both cancer and infectious disease targeted) [10, 12–18] that multiple subcutaneous immunizations with vaccines based on the new Ad5 [E1-, E2b-] platform induce CMI responses that were superior to those of current generation Ad5 [E1-]-based vaccines, can overcome the barrier of Ad5 immunity, and can be utilized in multiple immunization regimens requiring a generation of robust CMI responses. In our present report, the greatest magnitude of CEA-directed CMI responses occurred in patients receiving the highest dose of the vector. We observed that a CEA-directed CMI response was induced in a dose–responsive manner despite the presence of pre-existing and/or vector-induced Ad5 immunity. We did not assess CAP1(6D)-specific CMI responses in this phase I/II clinical study and plan to assess CAP1(6D) and other CEA epitope-directed CMI responses in our expanded clinical trials. No CEA-directed antibody responses were observed

either pre- or post-vaccination employing an ELISA technique [21]. In a preliminary analysis (data not shown), we also observed a population of polyfunctional CD8+ T cells (those that secrete more than one cytokine when activated) after immunizations, a sign of greater functionality of T cells induced by the vaccine. These data support the use of the Ad5 [E1-, E2b-]-CEA(6D) vector in homologous prime–boost regimens designed to induce and increase CEA-directed CMI responses in patients with advanced colorectal adenocarcinoma, as well as any number of other vaccine amenable diseases or applications.

Although the precise mechanism(s) of how the Ad5 [E1-, E2b-] vector platform accomplishes tumor antigen-specific immune induction in the setting of existing or induced Ad5 immunity is not fully understood at present, we believe there are factors that contribute to the favorable activity of this new platform. As compared to earlier generation Ad5 [E1-] vectors containing deletion in the early 1 (E1) gene region, the Ad5 [E1-, E2b-] vector platform with additional deletions in the early 2b (E2b) gene region exhibits significantly reduced inflammatory responses directed at the vector [11, 35, 36]. This can result in longer transgene expression and a reduction in elimination of transgene expressing cells (e.g., antigen-presenting cells) that would otherwise occur due to induced inflammatory responses [35, 37]. Since Ad5 late gene antigen expression is significantly reduced as compared to earlier generation Ad5 platforms [8, 11], this could enable the Ad5 [E1-, E2b-] platform to evade Ad5 immune-mediated neutralizing activity for significantly longer periods of time resulting in greater longevity and amplification of TAA expression. In addition, the E2b gene product, polymerase, is a known target of human cellular memory immune responses to Ad5 infection and its elimination from the vaccine could be furthering its capability in the setting of preexisting Ad5 immunity [38]. The extended and/or greater expression of TAA by the vector in this milieu could result in a more effective immune response against the target antigen. However, it is also possible that this vector configuration produces better transgene expression, different biodistribution, or different innate/adaptive immune effects that impact the effectiveness of this vector, rather than escape from preexisting immunity.

Our patient demographics, albeit limited in size, compares favorably with previously published studies of patients with chemotherapy-refractory colorectal cancer [39–41]. Of interest is the observation that treated patients in our study exhibited favorable survival probability. Overall, all 25 patients treated at least two times with Ad5 [E1-, E2b-]-CEA(6D) exhibited a 12-month survival probability of 48 % and this was achieved despite the presence of significant levels of preexisting Ad5 neutralizing antibody titers. However, the true impact of this new

immunotherapy on overall survival will only be determined in a statistically controlled and randomized trial with larger numbers of patients.

In other clinical trials, immunotherapeutic agents have been found to increase overall survival without having a direct impact on time to objective disease progression, a trend noted in our study as well [1, 42–44]. By engaging the patient's immune system, active immunotherapeutics, such as Ad5 [E1-, E2b-]-CEA(6D), could induce continuous immunologic anti-tumor responses over a long period of time that could result in a “deceleration” or alteration in specific aspects of the rapid growth rate or spread of the tumor not measured by standard response assessments [39, 45]. Indeed, we have observed slower tumor progression in Ad5 immune mice harboring established CEA-expressing tumors following treatment with Ad5 [E1-, E2b-]-CEA(6D) [12]. Moreover, it has been noted that overall survival might be the only true parameter for the determination of clinical efficacy of any potential cancer (immune) therapy [46].

As with any new treatment modality, safety is an important factor. In this phase I/II trial, we demonstrate that the Ad5 [E1-, E2b-]-CEA(6D) could be manufactured to scale, as well be easily and repeatedly administered by conventional subcutaneous injection techniques. The most common adverse effects were site of injection reactions and flu-like symptoms consisting of fever, chills, headache, and nausea. There was no impact on blood hematology or serum chemistries, and overall, the treatments were well tolerated. Specifically, no SAE were noted, and no treatments were stopped due to adverse events, indicating that a dose limitation to use of Ad5 [E1-, E2b-]-CEA(6D) in this clinical application had not been met.

These data suggest that patients with advanced colorectal cancer which are treated with Ad5 [E1-, E2b-]-CEA(6D) do not have serious adverse effects and may experience extension of life even if they have preexisting immunity to Ad5; however, this study had a small number of patients in a trial that was not randomized against a control population. The results of this trial are encouraging enough to advance to a large, randomized, single-agent trial. The observation that some of the patients experienced an increase in CMI which is dose dependent could be an indication that this may play a role in their clinical outcome. We plan to initiate a large multicenter trial which should give us the opportunity to evaluate in greater detail the influence of Ad5 [E1-, E2b-]-CEA(6D) treatment on safety, overall survival, time to progression following treatment, the levels of induction of CMI, and the relationship of induced CMI responses with clinical outcome.

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Conflict of interest The following authors declare financial conflict of interest: Elizabeth S. Gabitzsch, Younong Xu, Stephanie Balcitis, Rajesh Dua, Susan Nguyen, Joseph P. Balint, Jr., Frank R. Jones are employees of Etubics Corporation. All other authors do not have any conflict of interest.

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